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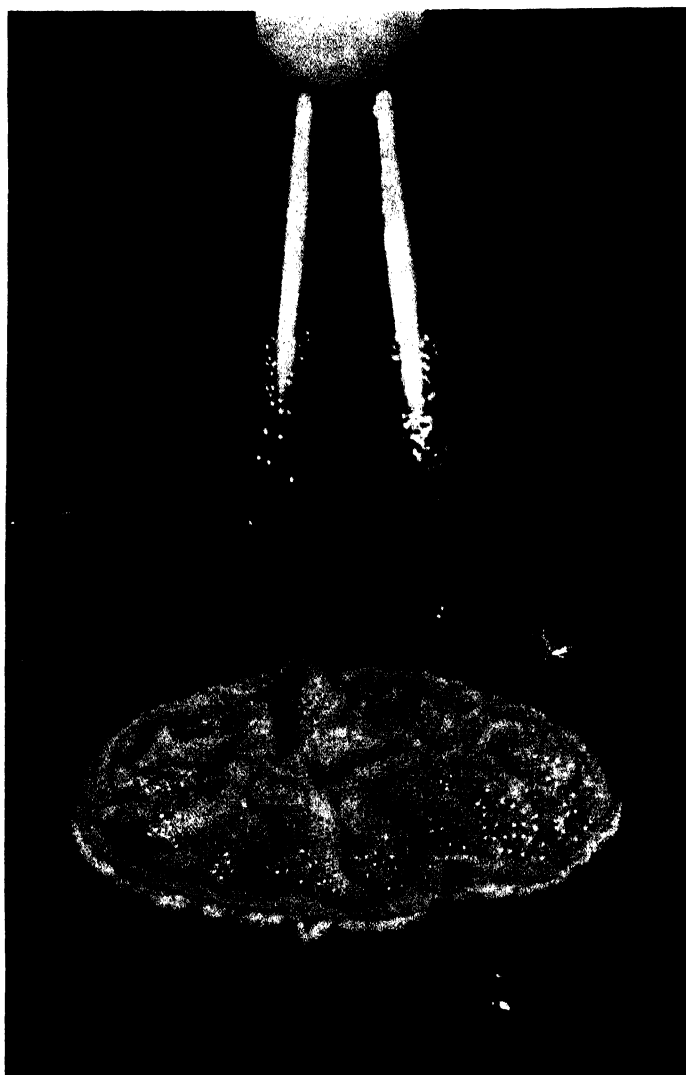
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The mold *Penicillium notatum* growing in a flask of culture medium for the production of penicillin. The thick, wrinkled mat of growth on top of the shallow layer of broth shows a green color on its surface due to countless spores. The yellow droplets on the surface are fluid exuded by the mold and are particularly rich in penicillin. The culture is about ten days old. Note the yellow pigment in the underside of the layer of mold. (See page 132.) (Courtesy of Merck & Co., Inc.)

# FUNDAMENTALS OF BACTERIOLOGY

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THIRD EDITION, RESET  
WITH 398 ILLUSTRATIONS

Philadelphia & London  
W. B. SAUNDERS COMPANY

comings of the text, it is felt that marked improvements have been introduced as a result. In preparing the chapter on pathogenic protozoa the author has had the expert and generous guidance of Dr. Marion M. Brooke, Associate Professor of Preventive Medicine, University of Tennessee, who is responsible only for what is laudable in that portion. Thanks are due to the staff of the W. B. Saunders Company for direct practical help of many sorts which only experienced publishers could give; to Miss Hermine Grimm, Editorial Associate of the American Journal of Hygiene, for untiring and faithful editorial assistance; and finally to Mrs. Frobisher, without whose aid, encouragement, and forbearance above and beyond the call of duty, little could have been accomplished.

Grateful acknowledgment is made also of the generosity of many scientists, publishers of scientific journals, and manufacturing concerns in furnishing and permitting the use of illustrative materials of unique value, the sources of which are cited in the book.

MARTIN FROBISHER, JR.

BALTIMORE, MD.

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# Fundamentals of Bacteriology

## CHAPTER 1

### INTRODUCTION—THE ORIGIN AND DISCOVERY OF BACTERIA

THE VISUAL experience of those to whom the microscope is unknown fails to encompass any living thing smaller than insects like fleas, lice and mites, or some of the algæ. The bacteriologist, however, has entrée into an amazing universe where those ignorant of the subject cannot follow. He becomes familiar with hordes of beautiful creatures, visible only beneath his lenses and, by special means, also becomes aware of the existence of mysterious, physiologically active structures so small as to be invisible even with the most powerful ordinary microscope but of immense importance industrially and medically.

Although bacteria are extremely minute, measuring usually about  $1/50,000$  of an inch in diameter and weighing as little as  $4/10,000,000,000,000$  (four ten trillionths) of a gram, it must not be thought that on this account they are unimportant or that they experience undue difficulty in the struggle for existence. Survival and persistence of a species do not depend on great size alone, but upon the power to live and multiply under a variety of conditions. Let us imagine creatures, for example, which can function in a range of temperatures from freezing to almost boiling, and regardless of whether free oxygen and food, as we know it, be present or not; creatures which, in addition to actively carrying on the business of life under these circumstances, can double their numbers every twenty minutes or, if required, cease growth and go into a state of what seems to be completely suspended animation, in this state surviving cold so intense as to liquefy hydrogen, extreme drought prolonged for many years, heat so intense as to coagulate blood, high pressures and high vacua. Let us imagine, furthermore, that the creatures are so minute as to be invisible to any enemies possessed of eyes with which to hunt them. Such creatures, it is clear, would be far more likely to survive the rigors of environmen-

tal change, and competition with larger forms of life, than unwieldy and relatively vulnerable structures like men, animals and trees. Such gifted organisms are the bacteria. Some of them are admirably adapted to survive (though not to multiply) under conditions such as those described, some of which probably existed in the early geological history of the earth; and to survive all the known vicissitudes of climatic change since those periods to the exclusion, if need be, of other, more advanced forms, of life. Not all species of bacteria possess all of the properties enumerated, but some of them do and these are among the most interesting creatures known. It seems not unlikely that, unless some grand cataclysm destroy all living things suddenly, such bacteria may be the last survivors of the pageant of life on this globe.

**The Primitive World.**—As we have seen, bacteria are very primitive structures and it may therefore be supposed that they represent the type of living beings which appeared very early in the earth's history of life. Indeed, for many years bacteria were regarded as probably the first cellular inhabitants of this planet. In attempting to form some idea of bacteria, their evolution and development and of the growth of our knowledge concerning them, it may, therefore, be of interest to review the story from its earliest inception, so far as fact and reasonable speculation may permit.

Let us discuss first the probable course of events after the earth came into being, attempting to imagine what sort of conditions the bacteria may have encountered. At first, the earth was probably entirely liquid; a mass consisting chiefly of molten metals surrounded by an envelope of superheated elements in a gaseous state. This gaseous envelope is believed to have been later composed largely of water-vapor, carbon dioxide, carbon monoxide, chlorine, hydrochloric acid and a small modicum of free nitrogen. Enormous clouds of water-vapor were continually boiling upward from the hot earth to the cooler, outer strata where the moisture was condensed. A steady downpour of hot, acid rain was the result. These rapid convections produced electrical tensions and the earth trembled under continuous and terrific detonations of thunder due to "lightning [which] linked, as with living, fiery tentacles, the cloudy heavens to the lurid, molten earth."<sup>1</sup>

This probably went on for millions of years. No life could have withstood the terrific heat. As time passed the tumult gradually subsided and eventually a relative silence ensued. The earth was sterile. A gradual cooling, with the formation of clinker-like crusts, and later with crystallization, followed. Water may have covered

the entire surface of the globe. Huge irregularities in the surface of the sea-bottom occurred and the water then collected into pools, lakes and oceans where it lay, rich in dissolved and suspended substances, including ammonia and ammonium salts, and warmed by the earth. The atmosphere, although still murky, was relatively rare and much cooler. Carbon dioxide was the predominating gas; nitrogen was present but oxygen almost absent. With subsidence of the earth's temperature to about 70° C., an environment suitable for certain forms of life, as we know it, finally existed.

The waters were rich in minerals and gases and substances in various states of *colloidal*\* aggregation, which were capable of forming compounds of an exceedingly labile and chemically active nature. These were doubtless the center of innumerable and varied physico-chemical reactions which must have been promoted by the then-existing conditions. These colloidal aggregations, we may assume, became more and more complex, some becoming so unstable as no longer to continue intact and therefore breaking down physically and chemically. Others were not broken down and combined with still more elements or molecules until globules of protein-like substance materialized out of the manifold solutes in the waters. These unstable colloidal compounds thus began to have some, *but not all*, of the properties of life. They may be imagined to have undergone a constant, slow, chemical change and to have retained, for a time, their globular and protein-like structure. As their size increased, they broke apart and thus multiplied.<sup>2</sup>

**From Nonliving to Living.**—This synopsis of the origin of life must not be viewed as entirely without basis. There are many facts which lend support to the idea as a whole, although there are so many gaps in our information that it is impossible to formulate a demonstrably correct story of the complete process. Oparin<sup>3</sup> has brought some interesting ideas concerning spontaneous chemical reactions to bear upon the subject. For example, he points out that at the high temperatures of the primordial world carbon would combine with other elements, forming hydrocarbons and cyanogen, as well as calcium carbide ( $\text{CaC}_2$ ) and other metallic carbides. These are demonstrable facts, and parallel phenomena are found in modern astronomical observations. Jupiter, for example, is be-

\* Colloids, for the purpose of this discussion, may be briefly described as substances existing as extremely fine particles or globules suspended in some fluid. Many elements and compounds can assume a colloidal state. Good examples of colloids are glue, egg white, milk fat and casein. Colloidal particles usually consist of several molecules of the same substance.

lieved to contain an island of heavy hydrocarbons floating on an ocean of heavier hydrocarbons, under clouds of ammonia and methane. Venus, it was recently suggested, is covered with great clouds of solidified formaldehyde. Organic compounds, then, can form spontaneously. Similarly, metal nitrides form at high temperatures. There is geologic evidence for this in the existence of such compounds as  $\text{Mg}_3\text{N}_2$ ,  $\text{CaN}_2$ ,  $\text{Al}_2\text{N}_2$ ,  $\text{FeN}$ , and so on, in volcanic material.

At high temperatures the carbides and N would react to form cyanamides ( $\text{CaC}_2 + \text{N}_2 \longrightarrow \text{CaCN}_2 + \text{C}$ ). Reacting with superheated steam these would produce ammonia ( $\text{CaCN}_2 + 3\text{H}_2\text{O} \longrightarrow \text{CaCO}_3 + 2\text{NH}_3$ ). Geochemical data show that such reactions actually occur.

Methane is found among the hydrocarbons on the atmosphere of Jupiter, and there is good reason to suppose that it existed also on the early earth. At  $1000^\circ \text{C}$ . methane changes to acetylene ( $2\text{CH}_4 \longrightarrow \text{CH}\equiv\text{CH} + 3\text{H}_2$ ) which, in the presence of  $\text{Fe}_2\text{O}_3$  and steam at  $300^\circ \text{C}$ . forms acetaldehyde ( $\text{CH}\equiv\text{CH} + \text{H}_2\text{O} \longrightarrow \text{CH}_3\text{CHO}$ ). Many similar reactions must have been occurring simultaneously in the primitive world, resulting in the formation of numerous organic complexes.

So the hiatus between the nonliving and the living is bridged by shrewd speculation. We imagine the gradual accumulation of the several attributes of life into colloidal particles, one by one, molecule by molecule, until we find our primitive colloids are no longer inert but possessed of a collection of chemical and physical properties which approach in complexity the phenomenon we call a living thing. This is not a cell as we know it, but merely a globule of complex, unstable chemical compounds held together by various physical forces, reacting together and with the environment, energy being obtained from electron transfers and various exothermic chemical reactions in the globule. Such primitive globules have been given the name "biococci."<sup>4</sup> Due to the dense atmosphere, solar energy was available only at much later periods and, as we shall see, most bacteria live best in total darkness and are soon killed by sunlight.

**Possible Origin of Bacteria.**—The following tentative statements on the nature of the hypothetical biococci may be postulated. They were minute specks or globules of a substance similar to chromatin (see p. 74). They were not limited by a rigid cell wall. Reproduction was by binary fission and their existence was vegetative. Various types of living things are believed to have evolved

from these biococci. First, the globule became surrounded by some sort of film or envelope. This resulted in a bacterium-like organism, the simplest form of which would be a sort of micrococcus. (Micrococci are spherical bacteria which seem to be no more than minute globules of chromatin surrounded by an envelope.)

Whether this history of the evolution of biococci and of simple forms of bacterial cells be true and exact in every detail, no one can say with certainty. Some such course of events, however, we feel safe in assuming to be the balance of probability after considering all the data available.<sup>5</sup>

The primitive bacteria of primeval times probably lived in the sea or mud, and from them probably evolved the saprophytic and pathogenic bacteria familiar to us today. Familiarity with bacteria and their properties, however, is of rather recent achievement since they could be studied accurately only after the development of lenses of sufficient magnifying power to make them visible. The rôle of the microscope in bacteriology, therefore, is important.

**The First Microscopes. Leeuwenhoek.**—Use of lenses has thrown much light upon all the questions which occupied the earliest biologists. By the end of the seventeenth century lenses had already been exploited in various ways for many years, Roger Bacon having used them experimentally before 1294. Such lenses, however, did not magnify very highly. The man mainly responsible for revealing the whole, hitherto unknown and unseen world of bacteria and other minute plants and animals, was the Dutch investigator (Fig. 1), *Antonj van Leeuwenhoek* (1632–1723 A.D.), a linen merchant by trade and a successful politician as well. Leeuwenhoek was an active, intelligent man of public and commercial affairs in the city of Delft. He was not a trained scientist but was self-educated. He amused himself by means of his skill and craftsmanship in glass blowing, fine metal work and other occupations. He came of a well-to-do and influential family and lived in relatively easy circumstances with plenty of leisure for his avocation of making minute lenses. With these he delighted in examining a great variety of objects. He examined saliva, pepper decoctions, cork, the leaves of plants, circulating blood in the tail of a salamander, seminal fluid, urine, cowdung, scrapings from the teeth and so on. In many of these he saw living creatures which we now know were bacteria but which he called “animalcules.” (“Animalcule” is a term which was used by the earlier workers to designate any minute plant or animal.)

His interest lay chiefly in his microscopes, however, and only

secondarily in what he saw with them, although the latter fascinated him. In spite of the fact that his microscopes were not compound he obtained remarkable results with them. "... he showed rare ingenuity and expert craftsmanship in the grinding and mounting of his simple lenses, a skill which he zealously kept to himself; and in spite of the requests of his learned friends, he refused to disclose the secret of his success." "... Leeuwenhoek's instruments are not true microscopes at all in the sense in which we think of



Fig. 1.—Antoni van Leeuwenhoek. A fanciful delineation based on a famous portrait. The picture shows accurately the size and shape of the first microscopes, the manner in which they were used, and the simple laboratory apparatus of the "Father of Bacteriology." (Courtesy of Lambert Pharmacal Co.)

microscopes, but rather simple magnifying glasses generally consisting of a small, single, biconvex lens. The object, and not the lens, was moved into focus by means of screws" (Fig. 2). "To adjust the lens to the object was so long and tedious a task that it is not surprising that Leeuwenhoek used an individual lens for each object. . . ." "The magnification varied and at best did not exceed two hundred to three hundred diameters." "The size of objects which Leeuwenhoek examined was determined by comparison. For this purpose he used at various times a grain of sand, the seed of

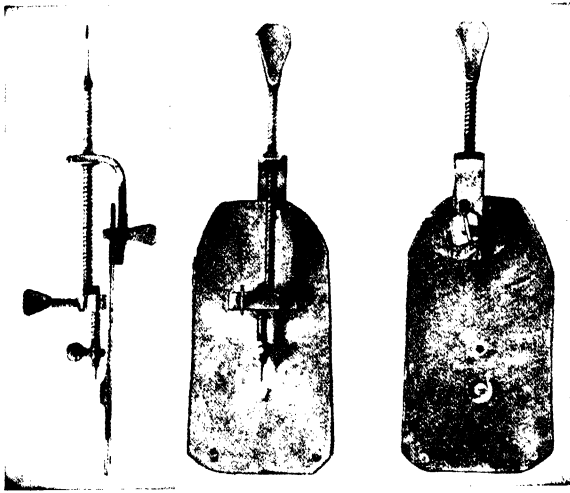


Fig. 2.—One of Leeuwenhoek's microscopes: front, back, and side views.

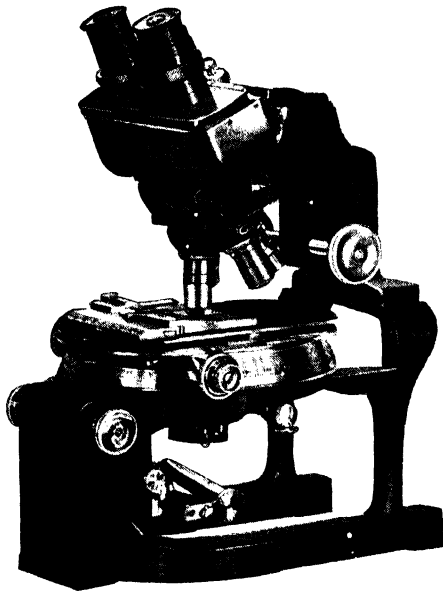


Fig. 3 —One of the most modern types of laboratory microscope, capable of giving clear images at magnifications of over 1000 diameters.



millet or mustard, the eye of a louse, a vinegar eel, and still later hair or blood corpuscles. In this way he secured fairly accurate measurements of a great variety of objects." ". . . he was forced to admit that the sand grain was more than one million times the size of one of the animalcules."<sup>6</sup>

Leeuwenhoek was so charmed with the things he observed that he felt constrained to write minutely detailed reports about them to the Royal Society in London<sup>6a</sup> and was later elected a Fellow of that body. Some of his observations are at once quaint and epoch-making. For example, after examining material which he scraped from between his teeth, he said, "Though my teeth are kept usually very clean, nevertheless when I view them in a Magnifying Glass, I find growing between them a little white matter as thick as wetted flour; in this substance, though I could not perceive any motion, I judged there might probably be living Creatures.

"I therefore took some of this flour and mixt it either with pure rain water wherein were no Animals; or else with some of my Spit-tle (having no Air bubbles to cause a motion in it) and then to my great surprise perceived that the aforesaid matter contain very small living animals, which moved themselves very extravagantly. The biggest sort had the shape of A (Fig. 4). Their motion was strong and nimble, and they darted themselves through the water or spittle, as a Jack or Pike does through the water. These were generally not many in number. The second sort had the shape of B. These spun about like a top, or took a course sometimes on one side, as is shown at C and D. They were more in number than the first. In the third sort I could not well distinguish the Figure, for sometimes it seem'd to be an Oval, and other times a Circle. These were so small they seem'd no bigger than E and therewithal so swift, that I can compare them to nothing better than a swarm of Flies or Gnats, flying and turning among one another in a small space."

**Natural Philosophy.**—Most of the men of Leeuwenhoek's time who were interested in problems of biology also interested themselves in questions concerning the origin of life. They were called students of *Natural Philosophy*. This included Zoology, Chemistry, Music, Astronomy, Geography, Art, Mathematics, Medicine, Literature, and so on. Medical treatises of that day were sometimes written in poetry and illustrated with fanciful pictures. When bacteria, through the agency of microscopes, were first brought within the range of human vision, they were viewed by students of natural philosophy from various standpoints. Some considered them the

original and lowest forms of life; others hailed them as the cause of disease. Furious debates arose, some of which still rage on. Those concerning the source of bacteria and their relation to the problem of the origin of life were especially vigorous and make interesting reading. Inasmuch as the history of bacteriology, and the lines along which it has developed are intimately bound up with these disputes over the origin of life it would be well for the student to devote a little time to some curious ideas that have been held on

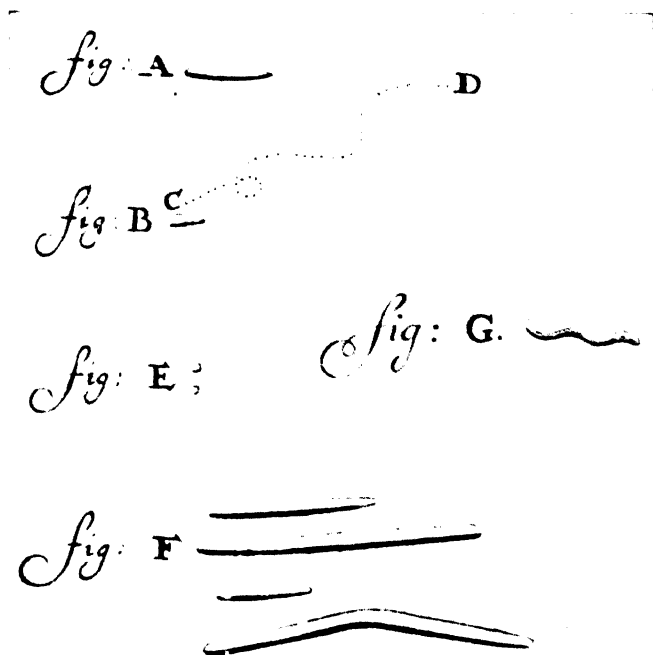


Fig. 4.—Leeuwenhoek's drawings of bacteria.

this subject. It is especially desirable to review some of these ideas since a number of discoveries were made, during the studies on the subject, which are still the basis of present-day bacteriology. Without a knowledge of these earlier discoveries, the student of bacteriology works at a disadvantage.

**Ancient Theories Concerning the Spontaneous Formation of Plants and Animals.**—The ancients knew nothing of bacteria and such primitive forms of life but believed that creatures like frogs,

mice, bees and other animals sprang fully-formed from fertile mud, decaying carcasses, warm rain or fog, and the like. Perhaps the student has read of Virgil's poetic recipe in the *Georgics* for producing swarms of buzzing insects from bullocks. "First, a space of ground of small dimensions is chosen; this they cover with the tiling of a narrow roof with confining walls, and add four openings with a slanting light turned toward the four points of the compass. Then a bullock, just arching his horns upon his forehead of two years old, is sought out; whilst he struggles fiercely, they close up both his nostrils and his mouth; and when they have beaten him to death, his battered carcass is macerated within the hide which remains unbroken. Then they leave him in the pent-up chamber, and lay under his sides fragments of boughs, thyme, and fresh cassia. This is done when first the zephyrs stir the waves, before the meadows blush with new colors, before the twittering swallow suspends her nest upon the rafters. Meanwhile, the animal juices, warmed in the softened bones, ferment; and living things of wonderful aspect, first devoid of feet, and in a little while buzzing with wings, swarm together, and more and more take to the thin air, till they burst away like a shower poured down from the summer clouds; or like an arrow from the impelling string, when the swift Parthians first begin to fight" (Newman).

*van Helmont* (1577-1644) devised a method for manufacturing mice. He recommended putting some wheat grains with soiled linen and cheese into an appropriate receptacle. Mice would then appear. This observation may still be *experimentally confirmed* but the conclusions drawn from it differ today. However an elderly lady of the writer's acquaintance complained bitterly that she had been cheated by a merchant who sold her a woolen coat which was of such a quality that it turned entirely into moths when left undisturbed in a closet for some months!

Such empirical theories of spontaneous generation of living beings were later discarded; but only after a most dramatic intellectual and experimental struggle, which will be detailed presently. All such theories assumed that life begins in an already highly developed state. It was later realized that life must have originated in a much simpler form.

After the development of the microscope an idea which held sway for some time was that life had its origin in the form of unicellular organisms usually thought of as having an amoeba-like structure and physiology. When one stops to consider the very complex nature of an amoeba, it is amazing that such an idea could

have persisted so long. According to Minchin, "It is impossible any longer to regard the cell, as seen in metazoa and as defined in the text books, as the starting point of organic evolution. It must be recognized that this type of cell has a long history of evolution behind it."<sup>4</sup> If we are to believe that life began according to natural laws we must look for something simpler than the cell from which more complicated creatures evolved. There has already been outlined a possible series of events which may represent the chemical evolution of "biococci," and it is possible that some such forms were the evolutionary precursors of more complicated cells.

**The Dispute Over Spontaneous Generation.**—In the earlier years, in the absence of exact knowledge of bacteria or chemistry and while Leeuwenhoek was amusing himself with lens making, there had already arisen much skepticism and bitter feeling over the question of the origin of life. One scientist who still held to the ancient ideas says of the views of another who doubted, "So may we doubt whether, in cheese and timber, worms are generated, or if beetles and wasps in cow dung, or if butterflies, locusts, shell-fish, snails, eels and such life be procreated of putrefied matter which is to receive the forms of that creature to which it is by formative power disposed. To question this is to question reason, sense and experience. If he doubts this let him go to Egypt and there he will find the fields swarming with mice begot of the mud of Nylus, to the great calamity of the inhabitants."<sup>7</sup> There was a great deal of such acrid discussion by wordy savants of the times who tried to settle everything by argument. Experimentation was regarded as rather undignified and even smacking of relations with the evil one.\*

**Francesco Redi (1626–1679).**—The experimental method was, however, being invoked by deep thinkers here and there. For example, it had always been supposed that the maggots in decaying meat were derived spontaneously from transformations of the putrid meat itself. Redi (Fig. 5), a poet and physician of Arezzo, placed meat and fish in jars covered with very fine gauze. He saw flies approach the jars and crawl on the gauze. He saw the eggs of the flies caught on the gauze and observed that the meat then

\* The student will note three main phases of the question of spontaneous generation:

(a) That concerning the origin of life through chemical evolution early in the history of the planet.

(b) That concerning the formation of complete and highly developed animals like bees, mice, frogs and the like from putrid matter, mud, cheese, dirty linen, etc.

(c) That which concerns the cause of putrefaction and fermentation.

putrefied without maggot formation. Maggots developed only when the flies' eggs were deposited on the meat itself. Redi's work was not widely noted, however, and it was not until much later that another series of experiments was made.

**Louis Joblot (1645-1723).**—After Leeuwenhoek's discovery it was thought by many who believed in the spontaneous generation



Fig. 5.—Statue of Redi in the Portico degli Uffizi.

of life that animal or vegetable matter contained a "vital or vegetative force," capable of converting such matter into new and different forms of life, and Leeuwenhoek's "animalcules" were hailed by many as proof of this. In 1710, Louis Joblot observed that hay, when infused in water and allowed to stand so for some days, gave rise to countless animalcules or infusoria (bacteria and protozoa). Many regarded this as conclusive evidence of spontaneous generation. Joblot, however, boiled hay infusion and divided it into two portions, placing one in a carefully baked and closed vessel, the other in an open vessel. The infusion in the open vessel teemed with living creatures in a few days. In the closed vessel no life appeared as long as it remained closed, thus showing that the infusion alone, once freed of life, was incapable of generating life anew spontaneously.

**John Needham (1713-1781).**—Similar experiments, carried out by an English scientist, John Needham (1749), gave conflicting results. Life developed in Needham's heated, closed vessels as well as in the open, unheated ones. He, therefore, believed in spontaneous generation. We shall see later that this sort of result was probably due to insufficient heating, which failed to kill heat-resistant spores. But nothing was known about spores at that time.

**Lazzaro Spallanzani (1729–1799).**—Spallanzani (Fig. 6), an Italian naturalist, published the results of a whole series of the same type of experiments, which disagreed with those of Needham. He showed that if dust and air are excluded with sufficient care, and if heating be prolonged sufficiently, and the vessels kept closed, no animalcules develop in hay infusions, or in any other kinds of organic matter like urine and beef broth. Needham, in reply, said the prolonged heating destroyed the “vegetative force” of the organic matter which, he said, was necessary for the spontaneous generation of life. Spallanzani answered Needham’s objections by the very simple expedient of showing that the heated infusions in the closed flasks could still develop animalcules *when exposed to air*.

In 1775 Lavoisier discovered *oxygen* and the relation between air and life and this renewed the controversy about spontaneous generation, the objection to Spallanzani’s results being raised that it was the exclusion of air (oxygen) from the flasks which prevented the development of life.

**Schulze and Schwann.**—New experiments were performed, in which air was admitted to the previously heated infusions of meat or hay, but only after passing through sulfuric acid or potassium hydroxide solutions (Schulze, 1836)<sup>8</sup> or through very hot glass tubes (Schwann, 1836),<sup>8</sup> their idea being that *the air itself introduced the germs of life into the infusions* (Figs. 7 and 8). When the infusions exposed to air so treated failed to develop any life, it was claimed by others that it was not due to a destruction of any germs of life in the air by the sulfuric acid or hot glass, but to the fact that the “life-giving” power of the air had been destroyed by these methods, thus preventing spontaneous generation. Schröder and von Dusch (1854–61)<sup>9</sup> overcame this objection by filtering the air only through cotton wool. This method prevented the appearance of animalcules in the heated broth or infusions until the ves-



Fig. 6.—Lazzaro Spallanzani.

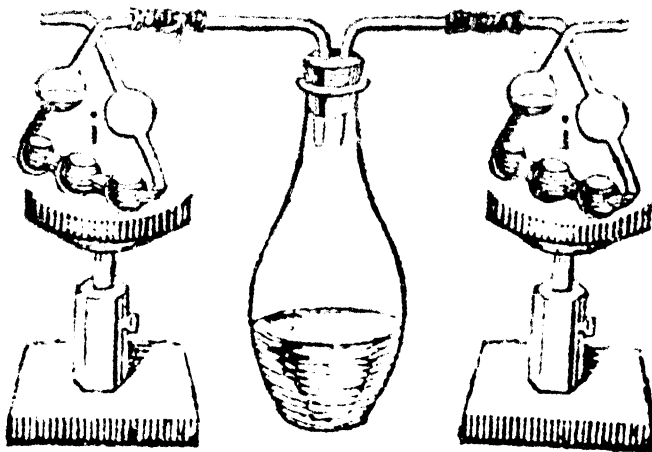


Fig. 7.—Apparatus of Schulze for treating air before admitting it to flasks of putrescible material.

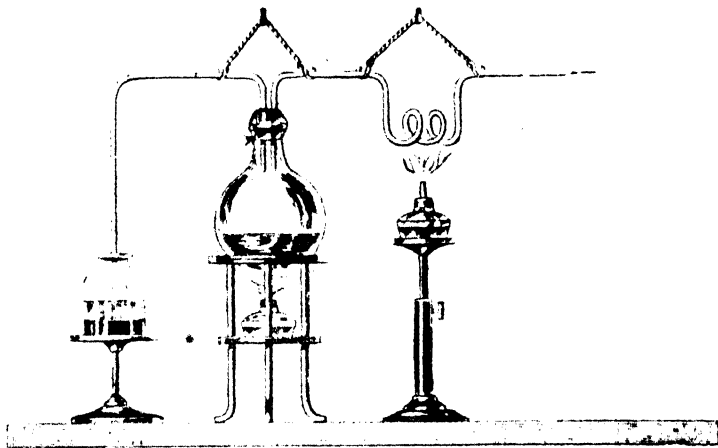


Fig. 8.—Apparatus of Schwann for treating air before admitting it to flasks of putrescible material.

sels were opened and it then became apparent that the method of treatment of the air had nothing to do with the development of animalcules, *and that these did not develop spontaneously* but that

there were particles of living matter in the air which not only were killed by heat, acids and alkalis but which could be caught and withheld by the cotton wool alone. The presence of the micro-organisms in the cotton wool was later proven by Pasteur. *The experiments of Schröder and von Dusch were the origin of our present-day use of cotton plugs for bacteriological culture tubes and flasks.*

In spite of these demonstrations long and bitter controversies still raged.

About this time four other scientists became intensely interested in the subject. These were Pouchet and Bastian, who supported the doctrine of the spontaneous development of "animalcules" or bacteria in infusions, and Louis Pasteur and John Tyndall, who opposed it. Pouchet was one of the bitterest of Pasteur's opponents. Space does not permit a long discussion of Pouchet's or Bastian's works, which were, in the end, completely refuted by the other two, but we must give some consideration to the life and work of Pasteur and Tyndall. Many of their discoveries are still in use in modern bacteriology and the details as to how the discoveries were made and the men who made them are of interest to all bacteriologists.<sup>10</sup>

**Louis Pasteur (1822-1895).**—Pasteur, the most famous, and considered by many the greatest, French scientist of all time, was born in Dole, December 27, 1822. Son of a moderately prosperous tanner who had fought for, and been decorated on the battlefield by, Napoleon, Pasteur had a great admiration for his father's soldierly accomplishments. He later was moved to many of his best scientific achievements by his patriotic zeal. In his boyhood he was an indifferent student but later became an enthusiastic scholar, devoting his energies to a study of chemistry. He discovered the relationship of the stereo-isomeric forms of tartrate crystals and revealed a whole new series of possibilities in physical chemistry.

Pasteur, however, was not one to gloat over such successes and rest on his laurels. He sought for other fields of investigation. His choice was guided largely by patriotic motives. An Englishman had said to him: "People are astonished in France that the sale of French wines should not have become more extended here [in England] since the Commercial Treaties. The reason is simple enough. At first we eagerly welcomed those wines, but we soon had the sad experience that there was too much loss occasioned by the diseases [souring] to which they are subject."<sup>11</sup> \* Germany was, at that time, making much better beer than France, and Pasteur under-

\* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.



took to make France a successful rival in that respect. In order to do so he made a long study of beer manufacture and of the cause of souring and spoilage ("diseases") of beer and wines. As a result of these studies he arrived at far-reaching conclusions. "Might not the diseases of wines," he said at the Académie des Sciences in January, 1864, "be caused by organized ferments; microscopic vegetations, of which the germs would develop when certain circumstances of temperature, of atmospheric variations, of *exposure to air*, would favour their evolution or their introduction into wines? . . . I have indeed reached this result, that the alterations ("diseases") of wines are coexistent with the presence and multiplication of microscopic vegetations" (Fig. 9). Pasteur had found that acid

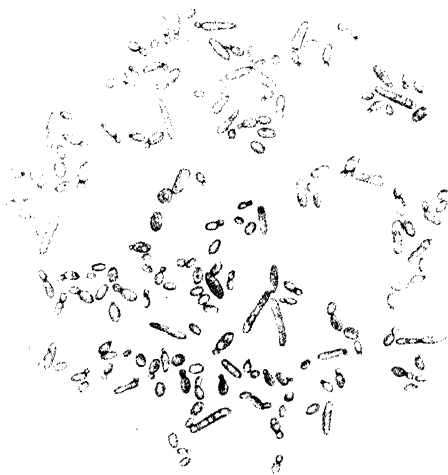


Fig. 9.—Yeast, drawn by Pasteur himself. (Pasteur, Translated by Faulkner and Robb, Macmillan & Co., 1879.)

wines, "ropy" wines, bitter wines, sour beer and so on, were due to the growth in them of undesirable contaminating organisms which produced these so-called "diseases."<sup>11</sup> \*

Pasteur soon became an authority on beer and wine manufacture and his advice and services were in great demand. "The town of Arbois, proud of its celebrated rosy and tawny wines, placed an impromptu laboratory at his disposal during the holidays of 1864; the expenses were all to be covered by the town."<sup>11</sup> \* Pasteur, however, fitted up his own laboratory.

\* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

The solution of the problem, according to Pasteur, lay in preventing the growth of foreign organisms, "wild" yeasts, bacteria, etc., which caused the undesirable conditions. After considerable experimentation along these lines (Fig. 10) he discovered that the diseases did not occur, and the wine did not spoil in transit, if it were held for some minutes at a temperature between 50° C. and 60° C. He said, "I have . . . ascertained that wine was never altered by that preliminary operation (heating), and as nothing prevents it afterwards from undergoing the gradual action of the oxygen in the air—the only cause, as I think, of its improvement with age—it is evident that this process (heating) offers every advantage." His experiments were so successful that a practical test of the efficacy of his methods was made. He wrote to a friend, ". . . experiments on the heating of wines will be made by the Minister of the Navy. Great quantities of heated and nonheated wine are to be sent to Gabon so as to test the process; at present our colonial crews have to drink mere vinegar."



Fig. 10.—Pasteur in his laboratory.

"A first trial was made at Brest on the contents of a barrel of five hundred liters, half of which was heated. Then the two wines were sealed in different barrels and placed in the ship *Jean Bart*, which remained away from the harbour for ten months. When the vessel returned, the Commission noted the limpidity and mellowness of the heated wine, adding in the official report that the wine had acquired the attractive colour peculiar to mature wines. The nonheated wine was equally limpid, but it had an astringent, almost acid flavour. It was still fit to drink, said the report, but it were better to consume it rapidly, as it would soon be entirely spoilt. Identical results were observed in some bottles of heated and nonheated wines at Rochefort and Orleans."<sup>11, \*</sup>

\* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

Pasteur laid down three great principles:

1. Every alteration, either of beer or of wine, depends on the development in it of microorganisms which are ferments of "diseases" of the beer or wine.

2. These germs or ferments are brought by the air, by the ingredients, or by the apparatus used in breweries.

3. Whenever beer or wine contains no living germs it remains unchanged.

In the same way that wines could be preserved from various causes of alteration by heating, bottled beer could escape the development of disease ferments by being brought to a temperature of 50° C. to 55° C. "The application of this process gave rise to the new word, 'pasteurized' beer, a neologism which soon became current in technical language." Today, pasteurization of milk (heating at 62° C. for thirty minutes) is commonplace. The heating kills the undesirable germs.

"Pasteur foresaw the distant consequences of these studies, and wrote in his book on beer—'When we see beer and wine subjected to deep alterations because they have given refuge to microorganisms invisibly introduced and now swarming within them, it is impossible not to be pursued by the thought that similar facts may, *must*, take place in animals and in man.'

It was obvious from Pasteur's studies that each special kind of fermentation or disease of beer or wine was the result of the growth and activity in it of a *special, distinct* form of yeast or other microorganism depending on the type of fermentation or disease under investigation. This furthered an idea, already old, of the *specificity* of biological action, and supported the view that animal and human diseases also, like different sorts of putrefaction and fermentation in wine vats, were each caused by a single, specific type of germ.

After Pasteur's views with regard to the nature of fermentation had been made public, he became involved in the bitter quarrel over the apparently mysterious appearance of the "germs" in fermentable or putrescible liquids like wine, beer, urine, broth, etc., hitherto regarded by many (Needham and others) as resulting from spontaneous generation. Without going into detail which would occupy too great a space, we may cite a series of experiments which Pasteur carried out to answer the various objections and fallacies of previous workers, and to show that the "animalcules" were merely descendants of germs which had gained access to the fluids from dust in the air and which, by their growth and metabolism,

caused fermentation and putrefaction. First he redemonstrated that living creatures float in the air attached to particles of dust. Then he showed, as Schulze and Schwann had done, that when they could be excluded from various things like sterilized broth and urine, these substances did not ferment or putrefy. By using flasks with long, open necks having several vertical bends in them, he showed that, although unheated and untreated and unfiltered air communicated freely with the interior, the dust was caught by gravity in the bends of the neck and no life appeared in the infusions (Fig. 11). Not until the flask was tilted so that the fluid came

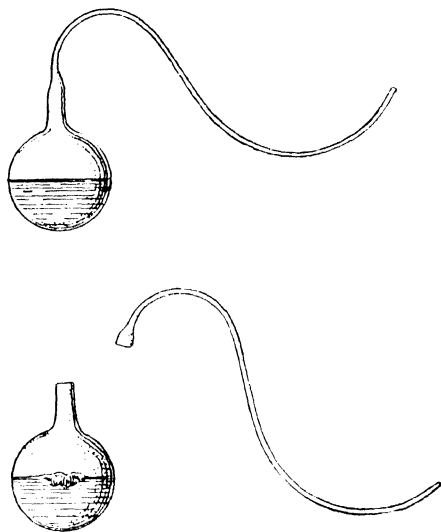


Fig. 11.—One of Pasteur's flasks, showing open neck with curves to catch dust (Duclaux, "Pasteur, The History of a Mind," W. B. Saunders Co., Philadelphia.)

into contact with this dust and was allowed to run back into the flask, or until the neck of the flask was broken off close to the body, did growth occur in the fluids. Some of Pasteur's flasks which were sterile in 1860 have been preserved and are still sterile (if they have not been destroyed by bullets) after 84 years! Nevertheless, Pouchet and Bastian still disputed loudly and long. The problem was taken up by the English physicist, John Tyndall.

**John Tyndall (1820–1893).**—Tyndall and Pasteur were friends. To investigate spontaneous generation, Tyndall arranged a small cabinet with windows in two opposite sides and in the front, a door in the back, and several holes in the bottom through which test

tubes were thrust. Openings were made in the top of the cabinet for the admission of air (through bent glass tubes like Pasteur's) and for the stem of a funnel. The interior of the cabinet was painted with glycerin which caught and held all dust particles as they settled so that after a time, when sunlight was passed through the cabinet from window to window, no "sun beams" (floating dust) could be seen (Fig. 12). The air was then said to be "optically pure."

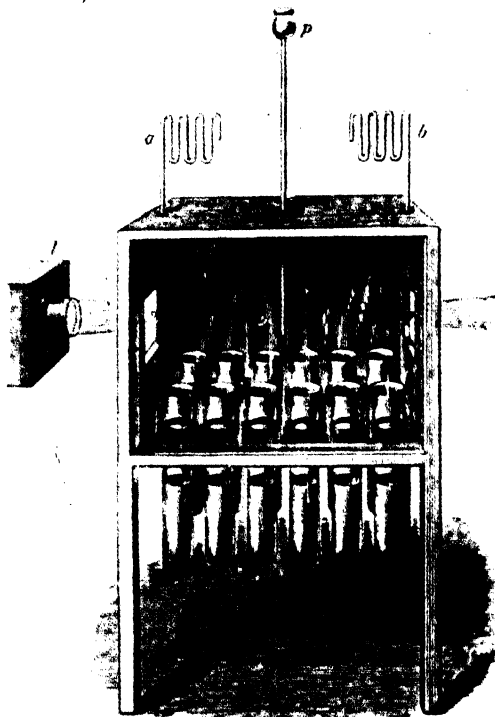


Fig. 12.—Tyndall's cabinet for experiments on spontaneous generation.

Tyndall now introduced mutton and vegetable broths into the test tubes through the funnel. The tubes were heated from below until they boiled for some time. They were left open and observed every day for many weeks. All remained sterile. They were perfectly capable of developing life, however, in spite of the heating, as shown by the fact that when dust was admitted to the cabinet all became putrid in a short time.

**Lord Lister (1827-1912).**—One of the most important applications of the work of Pasteur and Tyndall was made in 1867 by the English surgeon Lister (Fig. 13). He realized that wounds become infected, during surgical operations, by bacteria floating on particles of dust in the air, or by germs clinging to instruments or to the skin of the operator (Fig. 14). Obviously, in order to prevent such wound infections it was only necessary to render all surgical appliances sterile and, by antiseptic dressings and careful technic, completely to prevent the entrance of bacteria into surgical wounds. Lister's original method of preventing infection during surgical operations was to work in a field and atmosphere continuously wet with a fine mist of antiseptic, carbolic acid solution,



Fig. 13.—Lord Lister.



Fig. 14.—First illustration of amputation, from von Gersdorff (1517). Bandages constrict, at most, superficial veins. Two arteries are spurting uncontrolled. Man in background is wearing Gersdorff's pig's bladder dressing over his forearm stump.

emanating from a nearby apparatus (Fig. 15). This must have made the surgeon's work difficult but the results obtained were the foundation of our modern, aseptic surgery (Fig. 16). The student may judge for himself whether Lister's contribution was of importance to human life and medicine.

**The Beginnings of Precise Bacteriology.**—One reason, perhaps, why more species of bacteria were not discovered by Pasteur and others prior to about 1870 was that culture methods were very

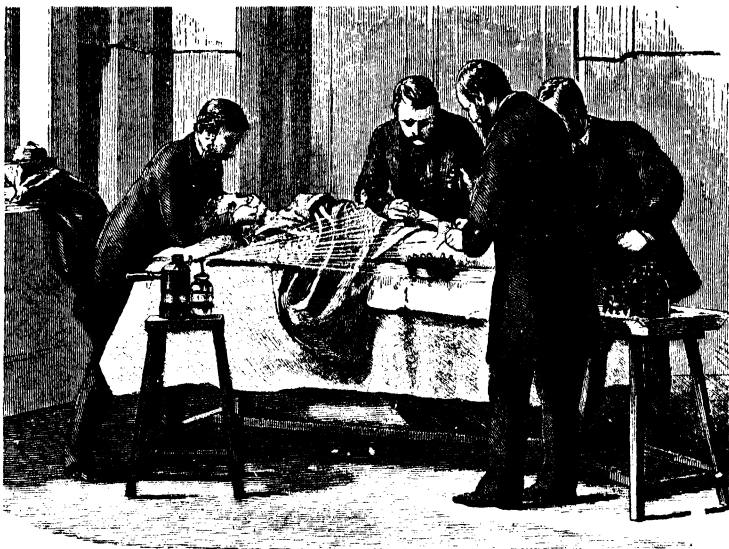


Fig. 15.—Lister operating with carbolic spray. Representing the general arrangement of surgeon, assistants, towels, spray, etc., in an operation performed with (supposed) complete aseptic (antiseptic) precautions (1882). Note the carbolic spray playing over the field of operation. (W. Watson Cheyne.)

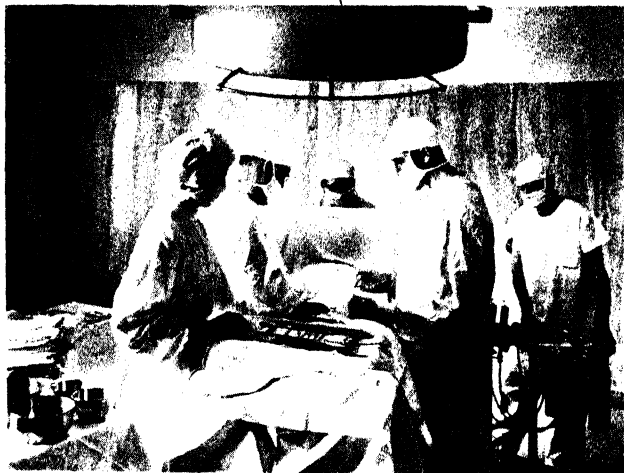


Fig. 16.—Modern surgery. Note the sterile masks, caps, gowns, rubber gloves, sheets. The instruments have all been sterilized. This is a U. S. Government Hospital. (Baltimore Sun, courtesy of A. Aubrey Bodine.)

crude. It was almost impossible, at that time, to separate, in a pure and uncontaminated state, any given sort of organism from a mixture of organisms. The preparation of such "pure cultures" is absolutely essential to the exact study of bacteria. For the development of pure culture technic, and for many of our modern bacteriological methods we are indebted to that precise German scientist, Robert Koch.<sup>10, 12, 13</sup>

**Robert Koch (1843-1910).**—While Pasteur, Tyndall, Pouchet, Bastian and others were engaged in their polemics and experiments on fermentation and spontaneous generation, Koch was practicing medicine as a sort of "County Health Officer" in Wollstein. He had occasion, in an official capacity, to investigate anthrax (a disease of animals and man caused by certain, cylindrically shaped bacteria of the form called *bacilli*) and he decided to study the disease in his laboratory during his spare time.

At first Koch and other bacteriologists examined all their bacteria in the living state, usually in drops of fluid mounted on a bit of glass. They thus became familiar with bacterial motility when present, and they observed refractile granules inside various bacteria. But the constant motion of some of the bacteria (either that purposeless oscillation, due to molecular impact and known as brownian movement, or real, progressive motion due to the action of flagella) as well as their transparency, made accurate and prolonged study most difficult. Koch realized that it would be much better for his drawings, and especially for his photographs (both of which, by the way, were excellent; Fig. 17) if the bacteria could be made to remain still. He tried spreading out his drops of anthrax-infected fluid in thin films and allowing them to dry, and met with immediate success. Not only were the anthrax bacilli motionless, but they apparently had not shriveled or changed in any visible way. However, the bacteria were transparent and colorless. It was very difficult to observe the fine details of their structure, and equally difficult to photograph them. He obtained ideas from other workers, a procedure commended to all investigators.

**Development of Staining Methods. Weigert and Ehrlich.**—Weigert, a German scientist contemporary with Koch, had observed the use, by Cohn and others, of various dyes to make clear the details of cell structures in histological preparations (histology = microscopic anatomy). This procedure had been in use for some time, the natural dyes *carmine* and *hematoxylin* being widely used. Ehrlich, a renowned chemist, had recently improved methods, discovered by William Perkin, of preparing very fine dyes from



coal-tar distillates. These were the first "coal-tar" or aniline dyes. Weigert, the bacteriologist, tried the methods of the histologists with the dyes invented by the chemist, Ehrlich. His first success was in 1875, when he found that the dye *methyl violet* could be used to reveal bacteria in histological preparations. This method of making bacteria easy to find and study, where before they had been colorless and transparent and therefore almost invisible, was



Fig. 17.—Koch's photograph of anthrax bacilli.

adopted by Koch and soon came into wide use. It is one of our best methods today.

**Development of Gram's Stain.**—In 1884, a Danish scholar, Gram, found that if anthrax bacilli were stained with Ehrlich's methyl-violet solution, and then treated with iodine, the dye was "fast" and that even alcohol could not remove it. In attempting to apply the method to certain other bacteria, however, he found that the dye was sometimes not fast and that as soon as alcohol was ap-

plied the color was removed and the bacteria were as colorless and transparent as ever. They could even be dyed an entirely different color with some other dye.

Upon further investigation it was found that all true bacteria could be divided into two great groups by this means. The former retained the violet stain when iodine and alcohol were applied, while the latter did not. When dealing with a mixture of the two kinds, a red dye could be applied after the alcohol and this would clearly bring out, in red, all the decolorized bacteria. They were



Fig. 18.—One of Koch's classes in bacteriology, 1881, in Berlin. Several of the men in this class became world-famous. Koch is the bearded figure near the center of the front row.

then seen in strong contrast to the purple ones. The bacteria retaining the purple stain were called gram-positive, while the others were called gram-negative. The method is used, generally with some slight modification, by every bacteriologist as one of the first steps in the process of identifying an unknown organism. Combined with an observation of the live organism in a drop of fluid it gives, to the trained bacteriologist, more information for less work than almost any other laboratory procedure.

Koch, using the methods of Ehrlich, Weigert, Cohn,<sup>14</sup> Gram,

Petri (see Chap. 7) and others, became one of the foremost bacteriologists of his day. His discoveries attracted scholars from all over the world (Fig. 18). In another chapter there is outlined the epoch-making development of solid media and the covered plate for the pure-culture cultivation of bacteria, which are methods used daily in every bacteriological laboratory in the world. The methods and principles which originated in the laboratories of Koch, Pasteur, Tyndall, Lister and others have made possible the conquest of disease and the building up of great industries. Greatest of all, they have left us a foundation of fact and inspiration upon which to build greater achievement and this, possibly, is the richest legacy which any man can leave to posterity.

**Other Developments.**—Pasteur, Koch and their contemporaries laid foundation stones of basic importance in the science of Bacteriology and their names are writ large in the Halls of Fame.<sup>15, 16, 17</sup> However, it must not be supposed that they were the last great bacteriologists. They were among the *first*. Other workers, equally ingenious, learned and courageous, have carried on the work along an ever-increasing number of lines of investigation. Although the five years following Koch's demonstration of the value of solid media for isolating bacteria witnessed the discovery of the organisms causing diphtheria, cholera, erysipelas, lockjaw, pneumonia and a number of other diseases, discovery of new species was only a matter of patient plating out of suspected materials and was only one, rather limited, phase of the subject.

Possibly the greatest single discovery in human history was made when, in 1890, von Behring and Kitasato found that guinea-pigs could be immunized against diphtheria toxin and that their blood then contained antitoxin which would protect other animals. The extension of this principle to other diseases was an obvious possibility and soon bore fruit.

In the realms of soil microbiology, great advances were going on simultaneously. In 1893-1894 Winogradsky demonstrated that atmospheric nitrogen is fixed in the soil by the anaerobic bacterium *Clostridium pasteurianum*. Aerobic organisms having a like property, *Azotobacter chroococcum* and *A. agile*, were discovered by Beijerinck in 1901. These organisms are of absolutely fundamental importance to the history of the human race since, without fixation of atmospheric nitrogen in the soil, there would be no human race at all. This rather deflationary fact will be explained later. Winogradsky, Beijerinck, Omeliansky and others during the decade following 1895 also worked out the processes by which the ammonia,

formed in the soil by various biological processes, is oxidized by successive stages, first into nitrites by organisms of the genera *Nitrosomonas* and *Nitrosococcus*, and then into nitrates by bacteria of the genus *Nitrobacter*. Only in the latter form is nitrogen available to agriculture and without it the human race would never even have gotten started.

In 1892 Iwanowski discovered the existence of a disease-producing agent (virus of tobacco-mosaic) which was invisible and not cultivable on inanimate media and which transversed filters capable of holding back the smallest bacteria. Many other viruses have since been discovered and great strides have been made in our knowledge of that class of organisms. For example, Walter Reed in 1900–1902 demonstrated not only the mode of transmission but the virus nature of the cause of yellow fever. Later, about 1933, workers of the International Health Division of The Rockefeller Foundation developed a fully effective vaccine against yellow fever and to date have administered it to nearly four million persons, chiefly in Brazil and to the men in the armed forces of the United States. The very development of such huge beneficent organizations as The Rockefeller Foundation, The George Williams Hooper Foundation and the National Foundation for Infantile Paralysis, sponsored by President Roosevelt, to mention only a few, are in themselves tributes to the continued and growing value of work in the fields of bacteriology and microbiology.

One interesting and exceedingly valuable series of discoveries has resulted more or less directly from the studies of Koch, Ehrlich, Weigert and the others with the staining of bacteria. From continued investigation of the effects of dyes upon bacteria there has arisen knowledge of such dye-like compounds as sulfanilamide and sulfadiazine, the use of which in treating bacterial infections is a matter of common knowledge.

So Science grows. The history of tomorrow is being written in the laboratory notebooks of students today. And who knows which notes will prove to be the basis of the greatest discovery? A small observation by Fleming in 1929 on a certain mold contaminating his cultures has borne fruit within the past three years in the form of penicillin, the famous new drug to combat infection (see page 132).

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to discuss briefly some of the properties of protozoa and to compare bacteria with these and with the blue-green algae and related

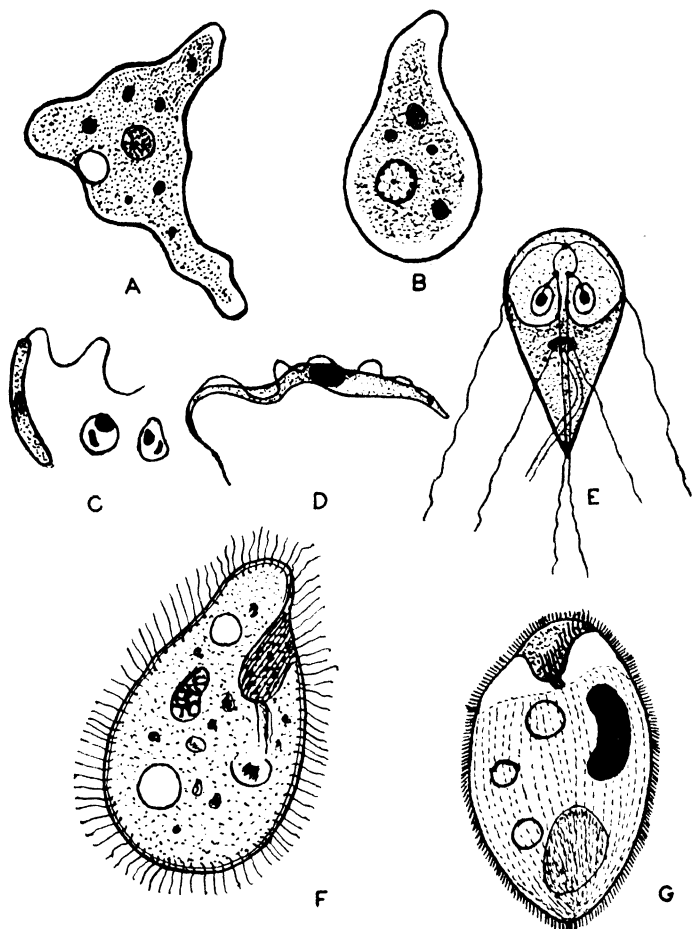


Fig. 19.—Various forms of protozoa. A, B, Forms of amoeba. C, D, Forms of trypanosome. E, *Giardia lamblia*. F, G, Types of ciliates. (After Hegner, Cort and Root, from Burdon, "Medical Microbiology," by permission of The Macmillan Co., publishers.) (See also Chapter 47.)

forms of life. It is assumed that the student already has a speaking acquaintance with both protozoa and algae and with the relations of these to more complex animals and plants.<sup>7-11</sup>

**Bacteria and Protozoa.**—Not all of the minute living beings which a microscopist observes are bacteria. In water in which a bit of hay has been allowed to soak for a few days, for example, not only do many kinds of bacteria abound, but also various other fantastic and marvellously beautiful creatures which resemble bacteria in many respects but which can, as a rule, be readily differentiated by their relatively huge size,

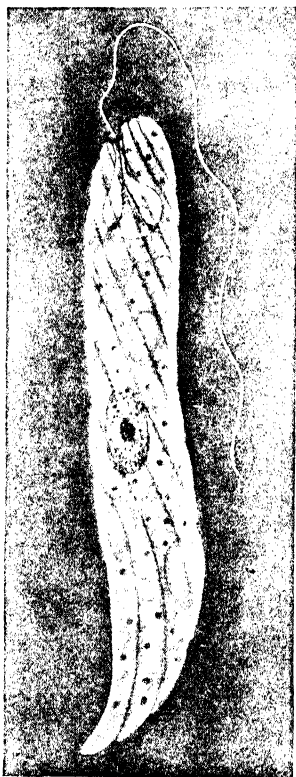


Fig. 20.—*Euglena* sp. (Hegner, "Big Fleas Have Little Fleas," published by The Williams & Wilkins Company.)

by their relatively huge size, their elliptical or ovoid form and other distinctive features. These are protozoa (Fig. 19)—microscopic animals each consisting of only a single cell. The protozoa are the most primitive members of the animal kingdom, just as most bacteria are the simplest members of the vegetable kingdom. It cannot, however, be properly said that protozoa are simple, as their one-celled body is often very complex and usually contains within it well defined portions performing the functions of organs in more highly organized animals.

The protozoa seem to be the results of development, by natural processes, of complexity of physiological function without increasing the number of cells. Thus, there is, in many free-living forms of protozoa, a portion of the cell which is somewhat thickened, rigid and even chitinous in composition. This serves as a skin or outer integument. This often contains or forms bristles (cilia or flagella) which have the functions of swimming appendages of higher animals. Although there are no proper muscles, there are certain portions of the protoplasm which are contractile and act the part of muscles in moving the cilia or flagella. The integument excretes waste products much as do the skin and kidneys of a human being, and thus serves the function not only of swimming oars, muscle and skeleton, but also of kidney, intestine and sweat glands. Inside the cell membrane there are usually a well-

defined nucleus, nucleolus and cytoplasm, the last often containing various vacuoles which collect waste products eventually to be excreted through the cell wall, thus serving the purpose of bladder and colon of higher animals. Other vacuoles contain food particles in the process of digestion and thus are analogous to stomachs. There are also granules of stored-up reserve material equivalent to fatty deposits or liver-glycogen reserves in mammals.

Although no such complex structures as nose, eyes, or nerves are found in protozoa, many of them are nevertheless quite sensitive to heat, chemicals, gravity, electricity, and light; and in one species at least (*Euglena*, Fig. 20), a portion of the cell is differentiated into a specially sensitive area called a "light spot" which, although not a separate organ, serves the function of a primitive eye. Furthermore, protozoa in general accept or reject food particles or waste, and have quite a delicate sense of touch so that they recoil on contact with hard objects, turning aside quite as though they were highly sensate and responsive creatures; all of which shows the marvellous possibilities contained within a single microscopic droplet of protoplasm. Reproductive processes are similar to those observed in the higher animals, inasmuch as there are, in many genera, sexual phenomena, with conjugation, nuclear interminglings, etc., in all essential respects analogous to the sexual phenomena related to fertilization in higher plants and animals. In protozoa, however, there are usually no special sexual organs.

One respect in which all single-celled plants and animals have an advantage over higher forms is that, for purposes of reproduction, an individual need only split itself in two. Among the flagellate protozoa this is usually done lengthwise, and each half receives half of each essential part of the original cell. Bacteria split transversely.

The cells of all living creatures possess this power of simple, sexless, or *asexual* multiplication. It is spoken of as *cell-fission*. In the unicellular forms, each cell remains a separate organism whereas, in complex forms, different cells group themselves together as into "guilds" or "unions" (the organs of the body, for example), each group performing one of the functions found in single-celled animals, but all the groups cooperating to form and maintain a complex society of cells, or organism, consisting of these large masses of cells having highly differentiated functions. In the single-celled organism cell-fission leads to the formation of new individuals; in many-celled organisms cell-fission generally adds only to the size of the individual of which the cell is a part

If we examine the animal kingdom as a whole, we find man at the top of the list, representing the latest and most complex development in organic evolution. Proceeding downward we pass through groups of lower animals less and less highly developed functionally and structurally and finally come to the protozoa, the least complex of all and the most primitive in an evolutionary sense. Only among the protozoa do we find any forms in the least resembling any of the bacteria.

Let us now examine the vegetable kingdom as a whole and consider the different divisions and their relations to each other and to the animal kingdom, especially the protozoa. Opinions differ as to the most logical method of classifying the organisms of the vegetable kingdom and no system can be said to be the best for all



purposes. For the present, the schema given below is a convenient arrangement.

### THE VEGETABLE KINGDOM

#### *Subkingdom I. Embryophyta* (leaves, stems, roots, flowers)

1. Spermatophyta (seed-forming plants)
  - Angiospermae (seeds borne in fruits)
    - Monocotyledoneae (1 seedling leaf; grass, lilies, etc.)
    - Dicotyledoneae (2 seedling leaves; bean, maple tree, etc.)
  - Gymnospermae (naked seeds)
2. Pteridophyta (ferns and allied plants)
  - Filicineae (ferns)
  - Equisitineae (horsetails)
3. Bryophyta (moss-like plants)
  - Musci (mosses)
  - Hepaticae (liverworts)

#### *Subkingdom II. Thallophyta* (without roots, stems, leaves or flowers)

1. Algae (contain chlorophyll; photosynthetic)
  - Rhodophyceae (red algae)
  - Phaeophyceae (brown algae)
  - Chlorophyceae (green algae)
  - Myxophyceae (blue-green algae)
  - Bacillariophyceae (diatoms)
2. Fungi (do not contain chlorophyll; rarely photosynthetic)
  - Lichenes (lichens)
  - Phycomycetes (alga-like fungi)
  - Ascomycetes (sac-forming fungi)
  - Basidiomycetes (club-forming fungi)
  - Schizomycetes (fission fungi or bacteria)

Subkingdom I may be disposed of as including only macroscopic green plants of relatively complex structure and showing well-developed sexual differentiation. None of these resembles bacteria or protozoa to any striking degree.

Subkingdom II contains many plants of relatively simple structure. Flowers and seeds are unknown. In spite of the fact that some of them (as the kelp-algae) may attain very large size (Fig. 21) (*Macrocystis*—600 feet in length) none is possessed of any well-differentiated root, stem or leaf structure, although certain sea-weeds approach this complexity. Sexual phenomena are usually absent or reduced to very simple forms. Some of the multicellular sorts consist simply of masses of cells clinging together in more or less characteristic groupings such as sea-weeds; each cell in the group being more or less independent of the mass and, if separated from it, often being able to produce a similar mass or “plant” by the simple process of cell fission, without the intervention of sex or any tendency of the cells to differentiate into organs as they in-

crease in numbers. Contrast this with the high degree of specialization in mammalian tissues, by imagining a bit of skin cut from your finger regenerating a complete duplicate of you!

Also occurring in each class and subclass are very minute and extremely simple plants in which even the primitive communal tendency described above is lost and each cell lives entirely disconnected from all others; each cell division resulting in a new indi-



Fig. 21.—A very large alga. A large kelp (*Pelagophycus*) from the California coast. (Photograph furnished by W. A. Setchell. Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

vidual, or at most in a loose pair, trio, or tetrad whose members are easily separated by mechanical agitation. Among the algae *Synecococcus*, *Gloeocapsa*, and *Scenedesmus* are examples of this form of plant (Figs. 22 and 23).

Some varieties of the green algae have cells (*Ulothrix* gametes Fig. 24) which resemble protozoa in form, size and motility, but differ in possessing the green coloring matter *chlorophyll*, which is common to these and all plants above the fungi. As a result of the

activity of chlorophyll the plants containing it synthesize materials of very complicated chemical structure (like starch and cellulose) from the simple substances carbon dioxide and water, absorbing energy from sunlight during the process and combining this energy as "caloric value" in the starch or wood (cellulose). The energy may later be liberated when the starch is used as food or the wood as fuel. This process of manufacturing complex energy-bearing substances from simple ones by means of chlorophyll using solar energy is called *photosynthesis*. Neither chlorophyll, nor its function of using solar energy to detach hydrogen from water in such a man-

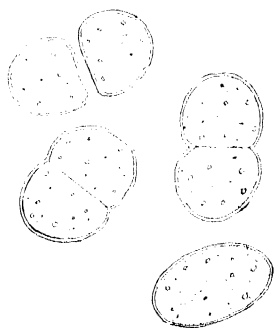


Fig. 22.—*Synechococcus*, a blue-green alga the cells of which are solitary except during fission. Several stages of fission are shown as well as a single cell about ready for division. (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

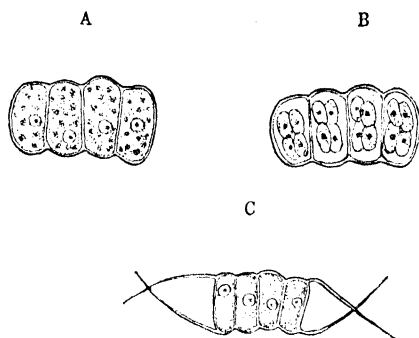


Fig. 23.—*Scenedesmus*, a simple green alga the cells of which form colonies of four (less commonly eight) cells attached side by side. *A* and *C*, two common species. *B*, the first of these giving rise to four new colonies. (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

ner that it combines with carbon dioxide to form substances that eventually become cellulose and starch, is found in the animal kingdom. There are a few exceptions such as *Euglena* and one or two borderline types.

Turning our attention now to the second group of *Thallophyta*, the fungi, we are struck by the absence of chlorophyll from all, excepting one or two families of bacteria which have a border-line status. In the group of fungi are organisms which so little resemble plants that they have no green color and are indifferent to, or even killed by, sunlight. The simplest of all of these are the bacteria.

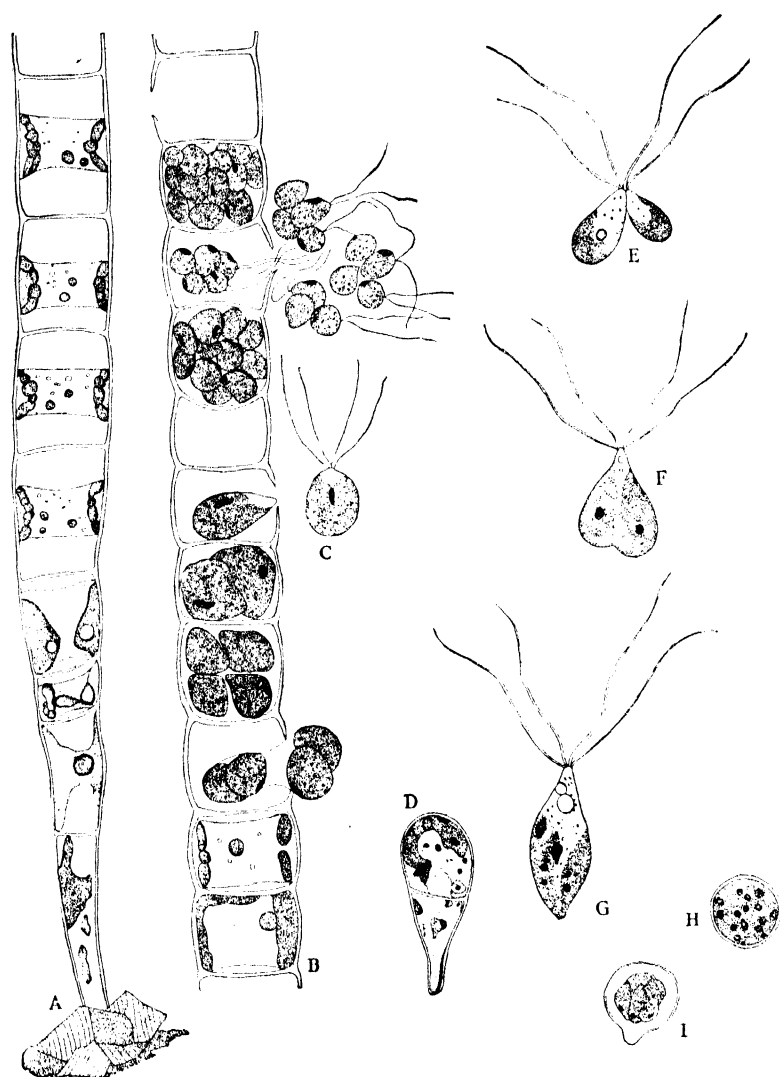


Fig. 24.—*Ulothrix*, a green alga showing motile, protozoan-like gametes. A. Portion of vegetative filament. B. Formation of gametes (above). C. Zoospore with four flagella. D. Germination of zoospore. E, F, G. Conjugation of gametes. H. Resting zygospore. I. Growth and germination of zygospore. (Reprinted by permission from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

If, as we may imagine, bacteria appeared very early in the earth's geological history, it is probable that they lived entirely in the dark



Fig. 25.—One species of "bracket" or "tree" fungus. (After Von Schrenk and Spaulding, in *Journal of Agricultural Research*.)

since it is believed that heavy clouds of vapor obscured the sun for many thousands of years until the earth became relatively cool.

At any rate, like other fungi, which probably evolved from bacteria, they lack the power of photosynthesis and make use of an entirely different source of energy to build up or synthesize their cell substances from simpler compounds. A wide variety of chemical devices for this purpose is found in different species of bacteria, but they have in common the fact that synthesis can proceed independently of solar energy, the built-in energy being derived from various chemical reactions rather than from sunlight. The process is therefore called *chemosynthesis*.

Among the fungi other than bacteria are found plants which are familiar because of their macroscopic size. The cells of most of them (except yeasts) are communal in habit, forming large masses



Fig. 26.—Various forms of bacteria ( $\times 900$ ).

of distinctive shape such as mushrooms, bracket fungi (Fig. 25) and puffballs, or smaller growths like the wooly molds seen on bread or decaying organic matter in warm weather. There are rudiments of sexual differentiation even in these low orders of life.

It is only when we descend to the bacteria that we encounter fungi which exist as single-celled individuals or at most only as small, easily broken up agglomerations of independent cells, devoid of sex, multiplying into individual plants by simple *fission*, and hence called the fission fungi or Schizomycetes (Fig. 26).

**Are Bacteria Plants?**—Although all bacteria are included in the vegetable kingdom, it is extremely difficult to justify this method of disposing of them *as a group* because so many of them possess

characteristics which are found in microscopic animals. Thus, we may say that bacteria are plants because their cell walls consist of cellulose, a characteristically vegetable substance. This is doubtless true of at least one (*Acetobacter xylinum*) and possibly of some other species, but it is certainly not true of all, or even of most. Some bacteria are said to possess cell walls of chitin, a distinctly animal substance which is the basic material of horn, hair, hoof and insect shell; but again, it is not generally true. The fact is that, because of the small size of the bacteria and the difficulties in their chemical study, exact information on this point is lacking.

Plants are said to differ from animals generally because the former are not motile while the latter are actively motile. With respect to large plants and animals and even among many of the very small forms this is generally true. Among bacteria, however, there are many motile as well as many nonmotile forms.

The simplicity of structure of the bacterial cell has been advanced as a basis for their classification as plants. This "simplicity," however, is merely a result of our inability to see the internal structures of the bacterial cell with ordinary microscopes. Photographs made with electron microscopes (see next chapter) reveal structural details of very great complexity indeed. Further, simplicity is not an outstanding character of plant cells.

The presence of starch in a living cell is, in itself, strong evidence of the vegetable nature of that cell, because the formation of starch is almost exclusively limited to the vegetable kingdom. On the other hand, glycogen, a starchlike compound, is almost exclusively limited to the animal kingdom, and cells containing it may be regarded as of an essentially animal nature. However, a polysaccharide which seems to be indistinguishable from glycogen is said to have been extracted from sweet corn. There are several common species of bacteria which build up granules of starch within their cells, and others which synthesize glycogen. Still others synthesize peculiar substances resembling starch, but neither true starch nor glycogen, and called "granulose" or "iogen." The composition of the reserve carbohydrate substance of bacteria is, therefore, only a source of confusion when advanced as evidence for the vegetable nature of all the Schizomycetes.

In some respects the diffuse nuclear structure of bacteria is thought to resemble that of the Myxophyceae or blue-green algae. This may be so but there is still controversy as to the exact nature of the bacterial nucleus, due to the limitations of available optical

methods. However, the bacterial nucleus certainly seems to be quite distinct from that found in cells of the animal kingdom.

**Manner of Taking Nourishment.**—All members of the vegetable kingdom (with few exceptions) differ from all animals (with few exceptions) in the manner in which food substances are taken into the body. Animals are able to take *solid masses* of food *into the body* (be it a many-celled or a single-celled body), there digesting it and turning it into soluble substances which nourish the cell or cells of the animal.\* This manner of taking food is called *holozoic*.† It is obvious enough in large animals, but may readily be observed in the smallest forms such as amoeba or paramecium with the aid of a microscope. With a few exceptions, like Venus's fly trap, the food of all plants, from the apple tree to the fern or sea-weed, is absorbed through the cell walls of root or thallus, and this absorption can occur only when the food is in the form of relatively *simple compounds soluble in water*. The plant cell cannot *engulf* solid food. This manner of taking food is said to be *holophytic*.‡

Holophytic nutrition is one universal property of bacteria which links them most strongly to the vegetable kingdom; yet even here we find that it is not an exclusively vegetable trait since some parasitic protozoa (trypanosomes, malaria parasites) and tapeworms are entirely holophytic. However, with these few exceptions, holophytic nutrition is probably the most generally vegetable-like property of the entire class of Schizomycetes.

The holophytic manner of taking food imposes some restrictions in the dietary of plants, and the chemistry of their metabolic processes is different in many respects from that of animals. Virtually all green plants can live on a wholly inorganic diet; a weak solution containing sodium nitrate ( $\text{NaNO}_3$ ), potassium phosphate ( $\text{K}_2\text{HPO}_4$ ), ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ), calcium chloride ( $\text{CaCl}_2$ ) and a half dozen or so other mineral salts combined with a little oxygen and carbon dioxide from the air serving very well for breakfast, dinner and supper; a life-long, never ceasing diet of salts! Yet from such materials we derive potatoes, watermelons, wood, paper, and thousands of drugs, paints, oils, perfumes and the like. A metabolic system adjusted to such a diet is said to be *autotrophic* or *prototrophic* and is a characteristically vegetable arrangement. Animals, in contrast, require complex organic substances derived from

\* If we consider the tissue *cells* of large animals, even this difference largely disappears.

† Holophytic means plantlike, holozoic means animal-like.



the bodies or wastes of plants or other animals, and this kind of metabolic adjustment is spoken of as *heterotrophic*.

Autotrophic metabolism (especially the use of inorganic carbon [ $\text{CO}_2$ ] and often other inorganic materials such as  $\text{H}_2\text{O}$ ,  $\text{NaCl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ , etc., as food) is probably the most distinctive vegetable trait, while heterotrophism (utilization of complex carbon sources) is a definitely animal characteristic; yet, while many bacteria are autotrophic, some of these same autotrophic forms can also live a distinctly heterotrophic existence, and many forms, especially disease-producing species, are limited to a heterotrophic diet exclusively.

The exact systematic position of bacteria in the animate world, therefore, seems to be somewhat doubtful at present. Probably some species should belong to the animal kingdom, some are undoubtedly vegetable in nature, while others may belong in an entirely new kingdom, neither animal nor vegetable—a sort of buffer state between the major taxonomic domains. However, it is not necessary that we settle the question before proceeding further with our studies.

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## CHAPTER 3

### HOW MICROORGANISMS ARE STUDIED OPTICALLY

HUMAN efforts to delve into the secrets of life are somewhat pathetic, equipped as man is, so very poorly for appreciating nature's subtleties. How much escapes his knowledge! Had he, for example, a special eye sensitive to infra-red, ultraviolet or other invisible rays; or some organ with which to detect cosmic radiations; or some receptor which could be stimulated by molecular motion or which could feel thought or hear radio waves, how much more he would know than he does or ever can! Will beings ever evolve possessed of these superior faculties? It is no more impossible than that man should have evolved from the primitive biococci which are thought to have preceded bacteria

Very early microscopists, about 1680, used simple, single lenses. These had definite limitations due to their crude construction. In attempts to see still more, better microscopes have been built,

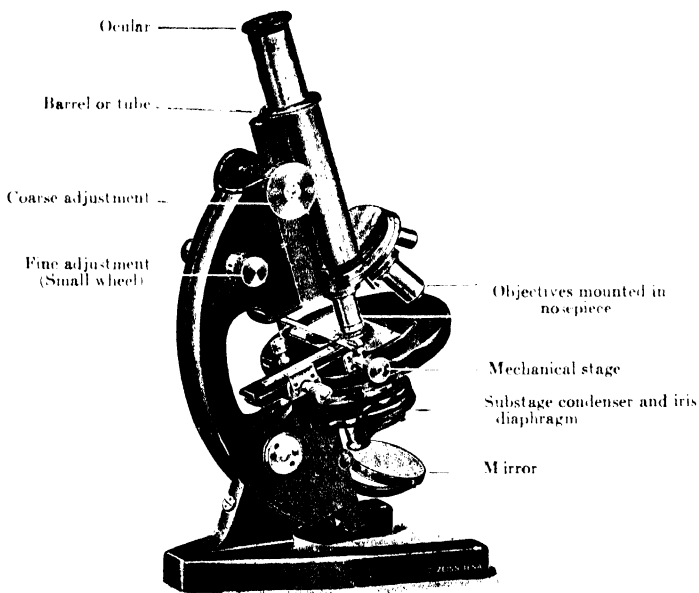


Fig. 27.—Microscope for bacteriological use.

until today we have very complicated, compound instruments which give magnifications of from two to ten times those of the best of the early instruments (up to 3000 diameters). Figure 27 shows a type of compound microscope in common use in bacteriological laboratories today.

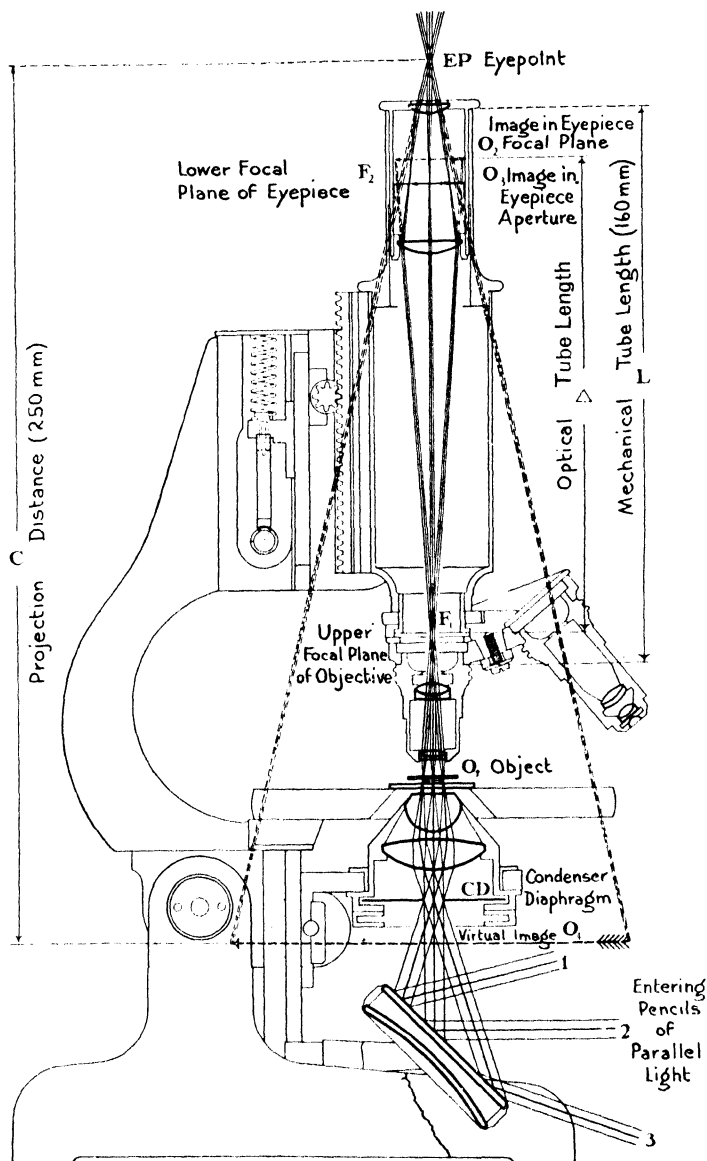


Fig. 28.—Chart showing path of light through laboratory microscope. (Courtesy Bausch & Lomb Optical Co., Rochester, N. Y.)

Complete description of the optical system of compound microscopes is not regarded as necessary here. Any good, modern college textbook of physics gives these details. However, a diagram showing the principal parts, as well as the path of the light rays through the instrument (Fig. 28), is included for the convenience of the student. A few directions for the use and care of the oil immersion lens may, in addition, not be out of place, as the bacteriologist uses this most of the time.<sup>1, 2, 3, 3a</sup>

An oil immersion objective lens magnifying about 90 to 100 diameters is generally used. The ocular or eyepiece should magnify 10 diameters. A total magnification of between 900 and 1000 diameters thus obtained is most useful, higher magnifications tending to give "fuzzy" outlines.

**Use of the Microscope.**—It is usually a little difficult for a beginner to obtain good results with an oil immersion lens. A reliable procedure is as follows: a bright source of light is needed, such as a 75 watt "frosted" electric lamp, a bright, unclouded sky (not direct sunlight) or some special form of lamp of which there are several types on the market.

Have the *stage* level. With the low power or the "high dry" lens centered about  $\frac{1}{8}$  inch above the condenser, apply the eye to the *ocular* and then adjust the *mirror* and *diaphragm* so that a maximum of light is thrown up through the *barrel* of the instrument. Raise the barrel of the microscope with the large wheel and swing the *oil immersion lens* into center, being sure it snaps into position. Put a drop of *cedar oil* on the smear to be examined. Mineral oil is to be avoided as a rule because, unless it has the same index of refraction as the glass of which the lenses are made, the image will be somewhat distorted. Some workers prefer it because it does not become sticky on drying, as does cedar oil. After applying the oil place the slide, *smear upward*, upon the stage, with the smear over the center of the *condenser* lens. Now, using the large wheel (coarse adjustment) lower the barrel, watching (*from the side*) the lowest point of the objective until it touches and is immersed in the oil and *almost* touches the smear itself. *It should not touch the smear.* With the eye at the ocular, very slowly *raise* the barrel with the large wheel until the smear comes into view. The small wheel may be used when the smear is very nearly in focus, or when it temporarily gets out of focus during manipulation. The mirror and diaphragm may now be adjusted more accurately. Sometimes it is desirable to close the latter a little to obviate glare and give more detail.

Never lower the barrel with the large wheel unless you are watching the objective tip closely; otherwise, you will mash the slide and may ruin the lens. If you have difficulty, it may be due to the fol-

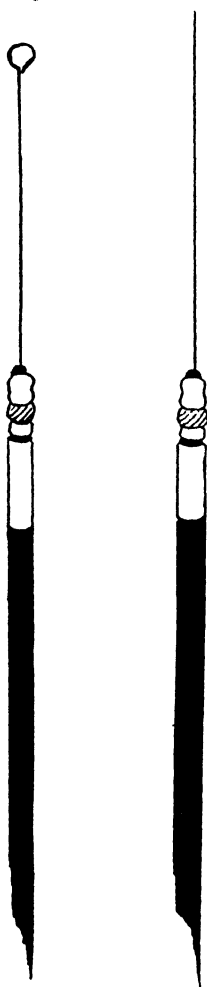


Fig. 29.—Wire loop and needle used for transferring bacterial cultures.

lowing causes: (a) slide upside down; (b) light insufficient; (c) bad adjustment of mirror or diaphragm or both; (d) use of the wrong objective or objective not centered; (e) dirty lenses (oil hardened on the objective should be removed with a piece of lens paper or soft cotton moistened with xylol); (f) poor focusing (try refocusing for practice); (g) condenser and substage not fastened up tight and close to the slide; (h) smear not under objective lens.

If you still have difficulty after checking up on these points, consult someone experienced in handling microscopes. Never attempt to repair your microscope or remove any lenses yourself. Keep your instrument clean and covered to keep out dust. Always remove immersion oil from the lenses immediately after use. Soft cotton, linen or lens paper should be used for this.

**“Hanging Drop” Preparations.**—Having become proficient in the use of the oil immersion lens, we may turn our attention to the things to be examined with it. If it is desired to examine bacteria in a natural, living state, they are best viewed when suspended in a clear fluid of some sort; usually water, saline solution or broth. The cells are transparent, colorless and refractile and so tiny that they are often difficult to find and even to identify as bacteria in the drop of fluid. This is especially true of the spherical types (cocci).

To observe bacteria in this state it is necessary to put a loopful\* (Fig. 29) of the fluid in which they are suspended, on a thin

\* By a “loop” is meant the space included by a tiny ring or loop made at the end of a thin wire. The wire is fixed into some sort of handle so that it may be sterilized in a flame. Loops are usually about 2 mm. in diameter and a loopful is a small drop.

cover slip. On each of the four corners place a droplet of mineral oil. Hold a "hollow-ground" slide, depression down, over the drop and bring the two into contact. Invert the slide quickly so that the drop cannot run off to one side. A *tiny* additional drop of clear mineral oil may now be run under the edges of the cover slip at each corner if needed. This spreads under the cover slip and prevents drying of the drop (Fig. 30).

In attempting to focus upon the drop, the diaphragm should first be closed about two-thirds. Bring the low power lens into center. The low power lens is useful to locate the edge of the drop, which is usually easiest to find and bring into focus. The high dry lens may then be brought to bear upon the drop, and then, if neces-

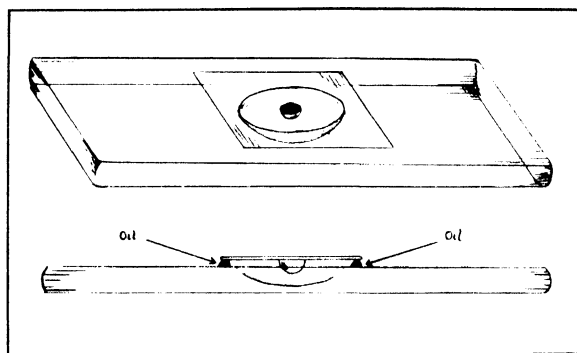


Fig. 30.—Hanging drop preparation. The size of the oil droplets is greatly exaggerated.

sary, the oil immersion. The latter is seldom necessary for inspection of the hanging drop.

In the hanging drop we may see the size, shape and arrangement of bacteria and their motion if they are motile. Sometimes bright, refractile granules and spores may be seen within the living cells. Due to the fact that bacterial cells contain no chlorophyll and are colorless and transparent it is extremely difficult to study them since this lack of color prevents details from showing clearly. Further, when suspended in fluid, they move about, either because of their own motility, currents in the fluid, or brownian movement. The observation of bacteria in hanging-drop preparations, therefore, yields only a limited amount of information.

**Preparations of Smears for Staining.**—To overcome this difficulty, Koch's device of immobilizing bacterial cells and coloring

them with aniline dyes is generally used. In order to accomplish this, the material containing the bacteria (soil, urine, pus, milk, feces, saliva) is diluted with water, saline solution or broth. A small drop of the suspension is spread evenly in a thin film, with a loop, over an area about 1 cm. in diameter. Sometimes a slide is marked off with a wax pencil into areas about 1 cm. square and then several smears may be stained at once. *Care must be taken that the material from one square does not run into adjoining ones.* A very subtle source of error is spattering. A beginner is prone to "dabble" his wire loop up and down in the wet smear he is making. This results in the formation of tiny droplets which spatter into nearby squares or onto the table, sometimes infecting people. Once the loop has touched the slide, make the smear with a horizontal rotary motion, and then lift the loop *slowly* from the slide *only* when the smear is finished. *Flame the loop* before putting it down. Flame it also just before use, but *be sure it is cool* (twenty to thirty seconds in air) before applying it to the bacteria.

Having made the smear, allow it to dry, and then heat slightly for two to three seconds over the Bunsen flame. This makes the bacteria adhere to the glass. The procedure is called *fixing*. *Do not overheat!*

**Staining Bacteria.**—For general bacteriological work basic stains (*i.e.*, compounds of color bases) are used, because bacteria react toward stains as though composed principally of nucleic acid, which takes basic dyes most readily.<sup>4, 5</sup> Thus, methylene blue, safranin, crystal violet, basic fuchsin, eosin, etc., are most generally used. All belong to the group of aniline (coal tar) dyes. Any simple aniline dye solution may be applied by flooding the smear with it. Löffler's methylene-blue solution\* is very widely used and reveals many details of form and structure. Other solutions and colors are also available for special purposes. The dye is allowed to remain in contact with the smear for about one minute and is then washed off with a gentle stream of cool water. The slide is then blotted (*not rubbed*) between two pieces of filter or blotting paper and when

\* Löffler's stain (Soc. Amer. Bact. "Manual of Methods").<sup>6</sup>

#### Solution A

Methylene blue .....	0.3 gm.
Ethyl alcohol (95 percent) .....	30.0 cc.

#### Solution B

KOH (0.01 percent) .....	100.0 cc.
Mix solutions A and B. The mixture keeps well.	

dry is ready for examination with the oil immersion lens as previously described.

Now, a simple stain such as Löffler's is of great value for many purposes. But another staining procedure, devised by the Danish scholar Gram, is more valuable because it enables us to differentiate kinds of bacteria, which may be of different species yet of the same general form and size. It is therefore called a *differential stain*.

**Gram's Stain.\***—To the smear which, for purposes of discussion, we shall assume to be of a substance containing a variety of bacteria (*e. g.*, saliva) and prepared as described above, crystal violet solution† is applied for 30 seconds. This is gently rinsed off and an iodine solution‡ is applied for 30 seconds. This, in turn, is rinsed off. Ninety-five percent ethyl (grain) alcohol is applied and renewed until all but the thickest parts of the smear have ceased to give off dye. (This usually takes from 20 seconds to 1 minute.)

The *differential* feature of the method is now apparent. Examination with the microscope will reveal the fact that, as Gram found, while many bacteria retain the violet-iodine combination, others will have yielded it entirely to the alcohol and are as colorless and as nearly invisible as before. Those species of bacteria *which retain the stain are called gram-positive*. Those *which yield it to the alcohol are called gram-negative*.

But the staining process is not yet complete. There is still the important final step of applying the *counterstain*§—a dye of some

\* **Gram's stain** (*Hucker's modification*, Soc. Am. Bact. "Manual of Methods")

Solution A	
Crystal violet (85 percent dye content).....	2.0 gm.
Ethyl alcohol (95 percent).....	20.0 cc.

Solution B	
Ammonium oxalate.....	0.8 gm
Distilled water.....	80.0 cc.

Dilute solution A about 1 to 5 with distilled water and mix with an equal volume of solution B.

‡ **Lugol's iodine:**

Iodine.....	1 gm.
Potassium iodide.....	2 to 5 gm.
Distilled water.....	300 cc.

Allow to stand 24 hours for the iodine to dissolve. It may be necessary to add a few more crystals of potassium iodide.

§ **Counterstain:**

Safranin (2.5 percent solution in 95 percent alcohol).....	25 cc.
Distilled water.....	75 cc.



contrasting color, usually eosin (red), safranin (red), brilliant green or Bismarck brown. Any one of these dyes colors the gram-negative species and they become as visible as the gram-positive ones, but are readily differentiated by their color.

Thus, by applying Gram's stain, which takes but 5 or 6 minutes, we can learn a great deal about any bacteria. We make visible not only the form and size and certain other structural details, but we can also at once assign the organisms which are present in the material being examined, to one of two great artificial groups of bacteria; the gram-negative or gram-positive. As will be seen later, this is very helpful in identification procedures. It should be noted, however, that some organisms are "borderline cases" in respect to Gram's stain, sometimes being positive, other times negative, and sometimes both positive and negative cells are seen in the same culture. As a rule, repeated tests will reveal the true nature of the bacterium. Often slight variations in cultural conditions or staining technic will affect the result. For example, many bacteria are not definitely gram-positive unless cultivated in the presence of at least 5 percent blood or serum. The reason for this is not clear.

As will be seen later the property of gram-positiveness is associated with other properties, especially sensitivity to certain poisonous substances such as sulfadiazine, *basic* dyes like crystal violet, and antibacterial substances of microbial origin, among them penicillin and gramicidin. Gram-negative bacteria on the contrary are markedly sensitive to certain other substances, such as *acid* dyes (sodium azide, *acid* fuchsin, etc.), salts like potassium tellurite and drugs such as sulfaguanidine and sulfasuccidine.

The reason for gram-positiveness in bacteria is not entirely clear. There is good evidence that the gram-positive material is located on the surface of the cells (in the genera *Bacillus* and *Micrococcus* at least) and may be removed like the bark of a tree, by appropriate procedures.<sup>7</sup> This is probably not the whole explanation however, and it appears that in many bacteria the violet stain is fixed inside of the cells by the iodine, in an alcohol-insoluble combination.

**Staining and Protein of Bacteria.**—Staining characters are in part related to the nature of the proteins of which bacteria are composed. Proteins are amphoteric compounds, combining with acids or alkalis. Aniline dyes may be of basic or acidic nature. Certain proteins, when in an acid medium, act as a base; if in an alkaline medium they act as an acid. Most bacteriological media are slightly alkaline. Bacteria composed predominantly of proteins which act as acids at the reaction of ordinary media will, therefore, combine

best with basic dyes. Basic dye is used in Gram's stain. Iodine tends to oxidize the protein and make it more acid so that it combines even more strongly with basic dyes. Thus bacteria which contain a large amount of acid proteins will tend to be gram-positive and the iodine used in Gram's stain will increase their affinity for the dye. In acid (fermented) media they will tend to be gram-negative. Gram-negative bacteria, on the contrary, must be supposed to consist largely of proteins which are not acidic in media of the usual alkalinity, so that they do not hold the basic dye of Gram's stain so strongly.<sup>8, 9</sup>

In addition to Löffler's and Gram's stains, there are certain stains designed to bring out special details such as spores, capsules, flagella and so on. Description of each of these methods will, however, be reserved to the discussion of the structural features of the bacteria to which it applies and to which we shall give some attention in the next chapter.

**Ziehl-Neelsen Stain.**—Another differential stain is that of Ziehl-Neelsen. It is used especially for staining tuberculosis bacilli in sputum and is applicable to all organisms having the chemical composition peculiar to these bacilli and closely related species. These are characterized by an abundance of waxy material in the cell. As a result of this waxy composition, when ordinary dyes like methylene blue are applied they fail to penetrate the wax and the bacilli remain unstained. By Gram's method they are stained purple but this method does not give as much information about them as the Ziehl-Neelsen or "acid-fast" stain.\* In using this stain a smear of the material to be examined is made as usual, dried, and fixed by heat. The smear is then flooded with the solution of carbolfuchsin and heated to 100° C., over a steam bath for 5 minutes. This softens the wax and the dye penetrates. After washing off the excess dye, the smear is treated for 5 minutes with cold 95 percent alcohol containing 5 to 10 percent hydrochloric acid. The wax re-

\* **Ziehl-Neelsen acid-fast stain** (*carbolfuchsin*):

Solution A

Basic fuchsin .....	0.3 gm.
Ethyl alcohol (95 percent) .....	10.0 cc.

Solution B

Phenol (melted crystals) .....	5.0 cc.
Distilled water .....	95.0 cc.

Mix solutions A and B. The mixture keeps well. A counterstain of Löffler's methylene blue is generally used, although some workers use brilliant green or saturated aqueous solution of picric acid for better contrast.

tains the red dye in spite of the acid-alcohol, which removes the color from everything else. If now, methylene blue or brilliant green be applied as a counterstain, the tubercle bacilli stand out as bright red bacilli in a blue or green field. The Ziehl-Neelsen stain is a *differential* stain because it differentiates acid-fast organisms from nonacid-fast ones (Fig. 31).

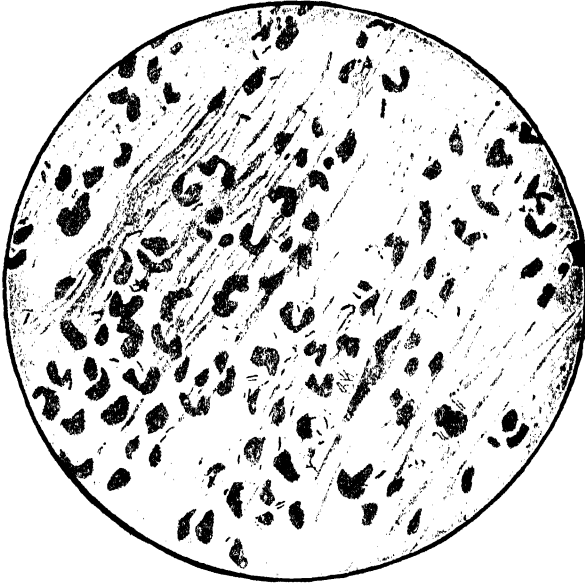


Fig. 31.—Tubercle bacilli in sputum. Ziehl-Neelsen; magnified 900 times. The bacilli have been stained red. The mucus and pus cells in the sputum are stained blue for contrast. Note the granular appearance of some of the bacilli, and their arrangement in clusters. The many bacilli pictured here are in a very tiny fraction of a droplet. (Cornet and Meyer.)

**Negative Staining.**—Another method of demonstrating bacteria is by means of a procedure referred to as “negative staining.” It really is not a method of staining bacteria, but of staining the background a solid black, usually with nigrosin,\* which fails to

**\* Dorner’s method:**

(*Grease-free slides must be used*)

Nigrosin.....	10 gm.
Water.....	100 cc.

Boil 30 minutes and add 0.5 cc. formalin.

Filter through paper and store in 2 cc. amounts in sterile tubes.

Place a loopful of the suspension to be examined on a slide.

penetrate the bacteria at all, and leaves them unstained to appear as light areas in the darkened field (Fig. 32). Only the outlines of the organism are made apparent by this method, and consequently it has a limited application, being used mainly for such forms as cannot be stained by any of the ordinary methods, especially spirochetes.

**Darkfield Method.**—A method analogous in principle and purpose to the “negative staining” procedure is that of darkfield illumination. The ordinary compound microscope may easily be equipped for this work. An opaque stop in the center of the sub-

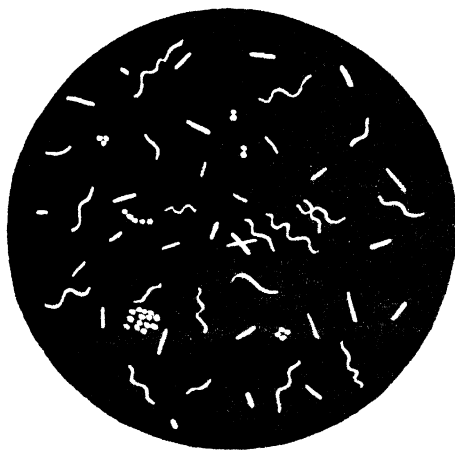


Fig. 32.—Appearance of various bacteria as seen when prepared by negative staining method ( $\times 900$ ).

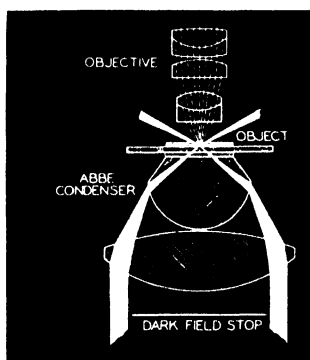
stage condenser is arranged to *prevent* the entrance of any *direct* rays of light from the mirror *straight upward* into the tube of the microscope. All *peripheral* rays are, however, reflected obliquely to the *center* of the upper surface of the condenser or microscope slide, emerging from the upper surface of the slide at such an angle that they do not enter the objective lens unless some object be present to *reflect* them *upward*. The empty field, therefore, appears dark (Fig. 33). When a fluid containing any particles such as dust, bacteria or spirochetes is placed on the slide at the focal point of these oblique rays, each particle becomes visible as a brightly il-

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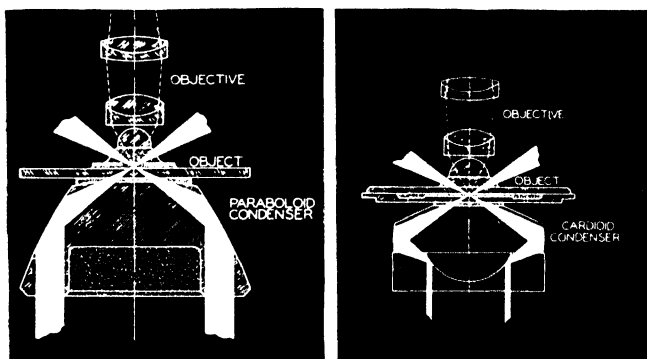
Immediately add an equal amount of the nigrosin solution, mix, and spread out in a *thin* film. (Thick films will crack and peel.)

Dry in air. *Do not wet the slide! Do not heat it!*

luminated speck due to the light reflected upward by it from its surface into the barrel of the microscope (Fig. 34). The remainder of the field appears black, hence the term *darkfield*. Unlike the process of negative staining, the darkfield shows not only outward



Abbe Condenser with  
Dark Field Stop



Paraboloid  
Condenser

Cardioid  
Condenser

Fig. 33.—Various forms of condensers for oblique illumination of the darkfield (Bausch & Lomb Optical Co.)

form but motility, since the organisms, covered with a cover slip, are examined in a moist, living state. As outward form and motility are the chief means by which certain important bacteria are distinguished, the method is very valuable in types of work where such species are involved.

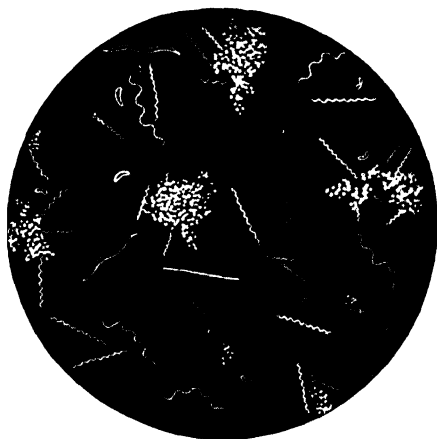


Fig. 34.—Fusospirochetal group of organisms, from a lung abscess. Note masses of cocci, long bacilli, small vibrios, and various types of spirochetes. Darkfield preparation ( $\times 900$ ).

#### ELECTRONIC MICROSCOPY

When Leeuwenhoek showed how to prepare and use lenses to investigate objects so small as to be entirely invisible to the naked eye, and reported his observations of protozoa, bacteria, etc., he was credited with one of the greatest contributions ever made to the advancement of science. As biological investigations continued, however, it became apparent, especially after the discovery of tobacco mosaic virus by Iwanowski in 1892, that there were creatures and objects so small as to make bacteria appear enormous by comparison and which could not be seen even with the most highly developed lens system.

The difficulty lay not with the lens makers but in the fact that the smallest wave length of visible light (blue-violet) is about  $4000 \text{ \AA}^{\circ}$  ( $\text{\AA}^{\circ} = \text{Angstrom Unit} = .000,000,01 \text{ cm.}$ ). Particles with a diameter of less than one half of this wave length or  $2000 \text{ \AA}^{\circ}$ , or lines separated by a distance less than about  $1800 \text{ \AA}^{\circ}$ , are not clearly visible, or become blurred, because of diffraction effects which cause interference of waves of  $4000 \text{ \AA}^{\circ}$  length. The oil immersion lens and compound microscope generally used by students magnifies only about 900 times. The best that can be expected of ordinary light microscopes is to reveal clearly details larger than  $.000,03 \text{ cm.}$  in diameter or somewhat over one half the shortest waves of visible light. Such details are relatively large as compared with many viruses. It is clear that advances in fields of investigation necessitat-

ing knowledge of *extremely minute* particles have been definitely restricted by limitations in optical equipment dependent on visible light.

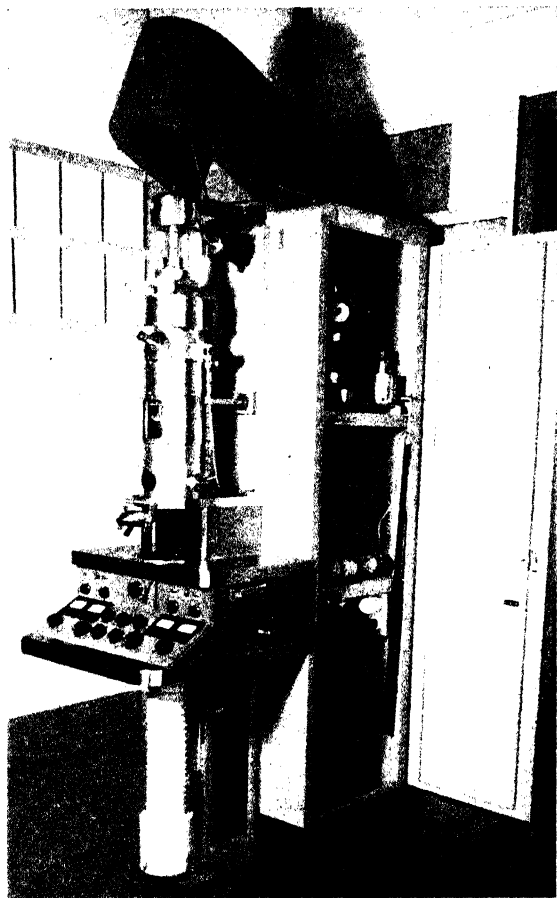


Fig. 35.—Advanced universal RCA Electron Microscope. The “heart” of the microscope is the stainless steel column (center) at the top of which is the electron gun. The diffraction camera unit is contained within this column, near the base. At the bottom of the column, just above the control panel, is the viewing chamber which has three windows so that several people can observe the image without the necessity of looking through port-holes as in the previous design.

While it has long been known that electromagnetic radiations such as x-rays have very short wave lengths, the preparation of glass lenses or reflectors for such radiations is impossible, so that

they cannot be exploited for optical purposes in the ordinary way. Although glass is opaque to electrons, two discoveries in the field of electronics have formed a basis for the use of electrons in micros-

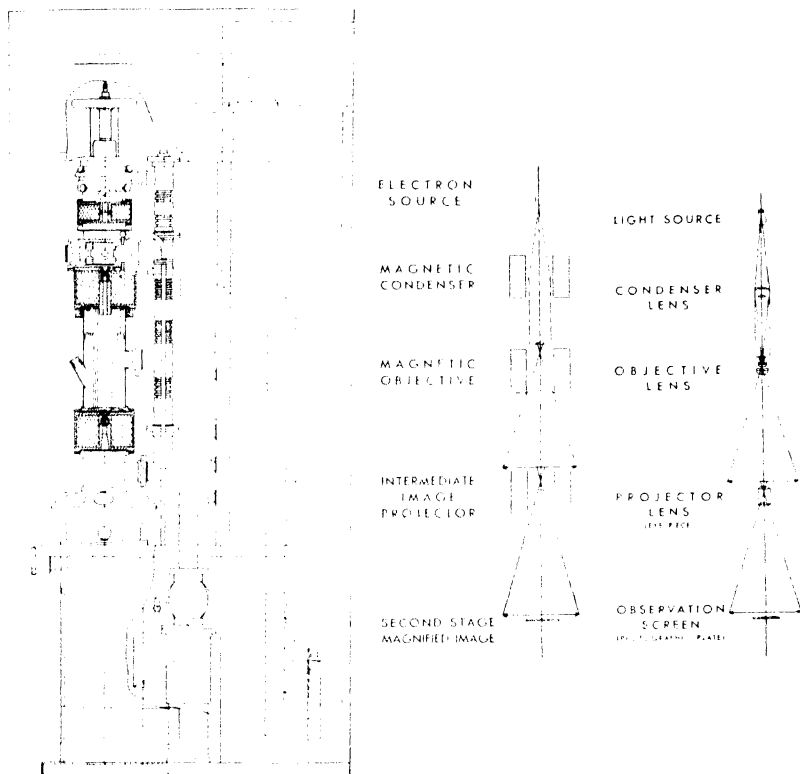


Fig. 36.—Simplified sectional view of the RCA Electron Microscope

Simplified sketch of the electron microscope developed in the RCA Laboratories at Camden, New Jersey. Diagram in center shows how closely analogous the operation of the electron microscope is to the conventional light microscope (right). Suitably shaped magnetic fields take the place of conventional glass lenses. A beam of electrons traveling at high velocity (at voltages of from 30,000 to 100,000) takes the place of ordinary light. The electron rays are converged on the specimen by condenser lens. After passing through the specimen, the objective lens coil forms a first image, enlarged about 100 times. The projection lens coil then magnifies the image again about 250 times, making an overall magnification of 25,000. The final enlarged image can be viewed directly by causing it to strike a fluorescent screen which makes it visible, or it can be made to record the image on a photographic plate for permanent record. The RCA Electron Microscope has such enormous resolving power that the final photograph can be usefully magnified by photographic enlargement up to 100,000 diameters.

copy. First, it was observed that electrons are deflected from their line of propagation by magnetic fields and, second, it was found that electrons moving with the speed imparted by 60 KV have a wave length of only about  $0.05 \text{ \AA}$ , or  $1/100,000$  that of visible



light. The latter discovery made plain the possible usefulness of electronic waves in microscopy, while from the former it was clear that circular magnetic fields could be used for "refracting" electron beams, much as a lens does light rays, forming electron images in the same manner that visible light images are formed by lenses.

From these two basic discoveries the modern electron microscope (Fig. 35) has evolved.<sup>10, 11</sup> The units in this instrument are nominally analogous with units in an ordinary compound microscope but deal with electron beams rather than light rays. The diagrams in figure 36 show the general arrangement of the focusing systems and other parts of an electron microscope compared with those of a light microscope.<sup>12</sup> The electron source is a tungsten filament at 30–100 KV potential. The electrons enter the magnetic condenser (analogous to an ordinary microscope condenser) and are concentrated on the object. They then pass through the object and enter the axially symmetric magnetic field of the objective magnet where they are focussed, and may be seen by interposing a fluorescent screen, the object being magnified about 100 times. The central (most clearly defined) area of this image is again magnified by an intermediate magnet corresponding to the "eye piece" of a light microscope and the image is projected into a second fluorescent screen or photographic plate corresponding to the eye of the student, with an added magnification of about 250 times (total 25,000). Portions of the photographs may be enlarged 4 times without undue loss of detail, thus giving a picture 100,000 times as large as the object. This degree of magnification is almost inconceivably great. A vague notion of it may be gained by imagining the page on which this is printed to be enlarged to the same degree. It would become a slab of spongy material as thick as a 10-story building is high (to get the effect of the height look down from the 10th floor), over  $5\frac{1}{2}$  miles wide and about  $8\frac{1}{2}$  miles long (to get the full effect of these measurements, walk the distance along a hot, dusty road).<sup>13</sup>

Several details of the operation of the instrument are of interest.<sup>13</sup> As the motion of electrons is impeded by air, the interior of the microscope must be maintained at a vacuum by means of suitable pumps. This necessitates air locks for the insertion and removal of objects and photographic plates (Fig. 37). The object mount is manipulated by a lever from outside the instrument which moves the object mount into a small chamber or lock which may be shut off from the main tube of the microscope. An object to be inserted is placed on the mount and moved into the lock which is closed and evacuated by an auxiliary pump before being opened to the main tube of the instrument. To remove the object, the steps are reversed. The lock is closed, air is admitted, the outer door opened and the object removed. The operator

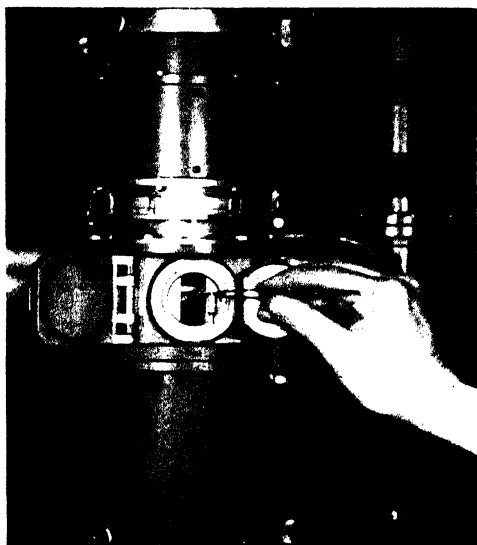


Fig. 37.—The Electron Microscope. Air lock and object chamber. (Courtesy RCA Corp., Camden, N. J.)



Fig. 38.—“Vest Pocket Size”—RCA has now produced this portable model of the Zworykin electron microscope which has made visible the secrets of the submicroscopic world. (Courtesy of Science Service.)

can look into the main tube, by means of portholes, and see the images on the fluorescent screens, manipulate the object, make suitable adjustments of field strength of the focusing magnets, alignment, etc. The object is mounted on an extremely thin (0.000001 cm. thick) cellulose film, since glass slides would be opaque to electrons. In newer forms (Fig. 35) of the instrument the details of vacuum manipulation have been much simplified, and other mechanical improvements installed.



Fig. 39.—The famed electron microscope, in console desk form, which makes this powerful explorer of the sub-microscopic world available to smaller laboratories, schools, hospitals, and factories, was shown for the first time at the wartime conference of the Society of American Bacteriologists by Dr. V. K. Zworykin (seated left). Opening unexplored regions to a host of research scientists, this new console electron microscope is capable of magnifying minute particles of matter up to 100,000 times.

The use of electron microscopes will hardly become general for some years, due to their cost and present restrictions on materials although a small electron microscope was developed in 1942 (see

Fig. 38). A development of this instrument is the small console model which is more convenient to use and more effective (Fig. 39). With the few instruments available, however, many exceedingly interesting studies have been made in the fields of chemistry, physics and biology with results of incalculable value. Crystal structure, colloids, organic molecules, film and filament structures, details of bacterial and other cell structure and many other matters hitherto hidden from us are now subject to our scrutiny. Several reproductions of electronographs (pictures made with electron microscopes) are found farther on in this book. Under conditions of electron microscopy living organisms cannot survive and physiological processes cannot be studied by this means at present.

A word of caution may be spoken in closing our discussion. The "objects" seen in photographs of electronic images are merely shadows, much like shadows on an x-ray plate, and depend for visibility only on degree of opacity to *electrons* of parts of the object. They may represent actual structures, or mere thickenings of the object, or artifacts. In any case, our interpretations of them should be extremely guarded until much more is known of electronic microscopy and its pitfalls.

### FLUORESCENCE MICROSCOPY

By fluorescence is meant the property possessed by certain substances of reflecting rays of a different wave length from that of the incident rays. Thus, substances having a certain color by ordinary light appear of a totally different color by ultraviolet light. Objects invisible when "illuminated" only by ultraviolet light may become brilliantly luminous if painted with a fluorescent substance such as quinine sulfate (fluoresces violet in ultraviolet light) or the dye auramine (fluoresces brilliant, luminous yellow in ultraviolet rays). By staining bacteria with a fluorescent dye and observing them in a field illuminated only with ultraviolet light, they can be made visible as brilliant, luminous objects readily seen and differentiated from nonfluorescent objects. This principle has been used for many years in microscopy.<sup>14, 15</sup> The dye coriophosphin has been used to demonstrate diphtheria bacilli by their specific fluorescent color, while protozoa and other cells, plant and animal, have been studied extensively by similar methods. Some cells contain naturally fluorescent substances.<sup>14</sup>

One of the most recent adaptations has been in the study of tuberculosis. The fluorescent dye, auramine, has a strong affinity for a wax-like substance, mycolic acid, in tubercle bacilli, and when the

bacilli are stained with auramine, and examined in the dark by ultraviolet light, they fluoresce with a luminous yellow light.<sup>16</sup> These facts have been taken advantage of in diagnostic work. Smears of sputum prepared as usual (see section on Ziehl-Neelsen stain) are stained for 2 to 3 minutes in a solution containing water, 97 cc.; phenol (melted crystals), 3 cc.; and auramine, 0.1 gm. The slide is then washed and decolorized for 5 to 10 minutes in a solution containing 70 percent alcohol, 100 cc.; concentrated hydrochloric

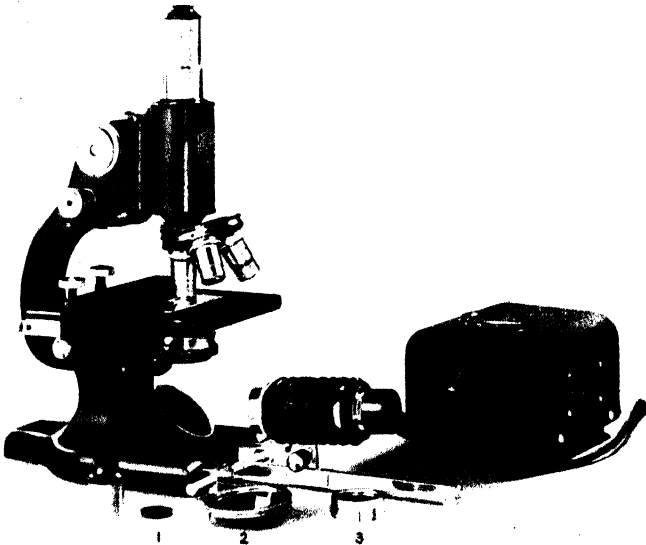


Fig. 40.—Attachments for fluorescent demonstration of acid-fast organisms. 1, yellow filter to go into ocular. 2, front surfaced, aluminized mirror fits over microscope mirror. 3, blue, ultraviolet transmitting filter for lamp. (Richards and Miller, *Amer. Jour. Clin. Path.*, 1941, 5 : 1 [Tech. Sect.])

acid, 0.5 cc.; and sodium chloride, 0.5 gm. The slide is placed on a microscope and examined in a darkened room by ultraviolet light. For light a carbon arc or a low-voltage, high-ampere, concentrated-filament lamp is to be preferred. The glass mirror of the microscope is covered with a polished aluminum mirror since the silver and glass would absorb too much of the ultraviolet light. A blue, ultraviolet-transmitting filter is placed over the lamp to withhold most of the visible light and a fully-complementary yellow filter is placed in the ocular of the microscope (Fig. 40) to permit the passage of

fluorescent light while withholding other light. If the filters are properly complementary no visible light, but only ultraviolet, will reach the eye and the field will appear black unless some fluorescent object be placed in it. In this case the tubercle bacilli stained with the phenol-auramine solution will be the only visible objects and so are easily seen and identified for diagnosis (Fig. 41). Objects not containing the mycolic acid (for which auramine has marked affinity) will have been decolorized by the acid alcohol and will not fluoresce and therefore will remain invisible. If the surrounding field is too bright, it may be decolorized for a longer time. Certain



Fig. 41.—Fluorescent tuberculosis bacteria in sputum smear. (Richards and Miller, *Amer. Jour. Clin. Path.*, 1941, 5 : 1 [Tech. Sect.])

impurities on the slide or in the sputum smear may cause nonspecific fluorescence.<sup>17</sup>

The short wave length of ultraviolet light (3500 Å°) may also be used to prepare highly magnified photographs of particles much smaller than bacteria and invisible with ordinary light. The material to be examined is first brought into focus with a darkfield apparatus and ordinary light, and is then photographed with ultraviolet light. A darkfield condenser of quartz and special reflecting surfaces must be used for the latter purpose, with an ultraviolet-rich light source. The electron microscope will supplant this procedure.

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## CHAPTER 4

## MORPHOLOGY AND STRUCTURE OF BACTERIA

**Morphological Types of Bacteria.**—As soon as microscopes and microscopy were sufficiently well developed to permit detailed studies of bacteria, it was found that several forms of these organisms could be distinguished (Fig. 26). Two main forms may be differentiated, the spherical and cylindrical.<sup>1, 2</sup>

*Spherical bacteria* are called *cocci* and these are classified into

several groups, mainly on the basis of the manner in which they cling together after fission. First, there are *diplococci*, which remain in pairs. Obviously these divide in one plane. Then there are the *streptococci* which, like the diplococci, divide in one plane but which cling together in chains, looking much like strings of beads. Other cocci divide in two planes, and cling together irregularly in masses shaped like bunches of grapes. These are called *staphylococci* or *micrococci*. Still others divide in three planes at right angles to each other forming cubical groups. These are named *sarcinae*. The spherical form of cocci is often distorted by various influences, so that oval, elliptical, conical and other modifications are frequently seen.

*Cylindrical bacteria* are of two main types: straight, rod-like forms collectively spoken of as *bacilli*; and curved or spirally twisted types, of which there are three principal subdivisions as follows: Those which are much like bacilli in shape but merely curved are placed in a genus called *Vibrio*. Those which are spirals of one or more complete turns and which are *rigid*, like a twisted stick of wood, are placed in the genus *Spirillum*. Those which likewise are spirally twisted but which are also *flexible*, like a piece of thin, coiled wire, are grouped in the order of *Spirochaetales*, collectively referred to as spirochaetes.

The relations of the morphological types may be clarified by the following outline:

*Spherical (cocci)*

Diplococci (pairs)  
Streptococci (chains)  
Staphylococci (irregular groups)  
Sarcinae (cubical groups)

*Cylindrical*

Bacilli (short, straight rods)  
*Vibrio* (short, curved rods)  
*Spirillum* (spirally twisted, rigid)  
Spirochaete (spirally twisted, flexible)

Examples are seen in Figures 26, 32 and 34.

As bacteria grow they are subject to many influences which affect their size and shape, much as various factors affect the size and shape of potatoes or peppers. Age also plays its role.<sup>3</sup> Individual differences in such respects are seen in all bacteria.

Referring to the straight, rod-shaped, cylindrical forms, it should be pointed out that the term "bacterium" was originally employed for all. However, when endospores\* were discovered by Cohn in

\* Endospores are oval, refractile protoplasmic bodies which develop inside of certain kinds of bacterial cells. Only one is formed in each cell. It represents a dormant stage which is quite resistant to drying, heat, disinfectants, etc., and enables bacteria to survive conditions unfavorable to growth. Spores will be discussed more fully later.



1875 in the rodlike species of aerobic bacterium now known as *Bacillus subtilis*, he differentiated these sporulating organisms from the other cylindrical bacteria and adopted the term "*Bacillus*" as a genus name. For this reason, the term "bacillus" should be employed only for aerobic rod-shaped species which produce true endospores. The term "bacterium" is applicable to rod-shaped species which fail to produce these bodies. In general, the terms "bacillus" and "bacterium" are used interchangeably for all rod-shaped cylindrical forms. The term "bacterium" is also used in a general sense to mean any organism belonging to the class Schizomycetes.

**Size of Bacteria.**—The drawings and reports by Leeuwenhoek (Figs. 1 and 4), the first to observe and describe bacteria (*circa* 1700), gave men an idea of the size and form of certain common types of these organisms.

Their minute size may be emphasized by various comparisons. For example, it is estimated that a cubic inch would hold nine trillion (9,000,000,000,000) cells of a medium sized bacillus (*Eberthella typhosa*); while four hundred million (400,000,000) would only occupy the volume of a "granule" of granulated sugar. It is common practice to magnify bacteria nine hundred to one thousand diameters with microscopes. They may then look no bigger than a period on this page, or an exclamation point (!). A man magnified to the same degree would be over a mile high and 500 yards wide.

The size of bacteria ranges from a diameter of about 0.5 micron\* (0.0005 mm.) and a length, in the cylindrical forms, sometimes as small as 1 micron (0.001 mm.). Lengths of 200 to 500 microns (0.2 to 0.5 mm.) are not unknown, however, in certain species.

**Bacterial Anatomy.**—Details of bacterial anatomy are not all clear. Although minute granules and rods of various size and arrangement are often demonstrable inside bacteria, their nature is obscure. They are variously regarded as nuclear material, "protoplasmic concentrations," degenerated material or food material. Nothing definite is known concerning a nucleus or any other internal structures. Even the presence of a chemically differentiated cell wall is in doubt although the electron microscope has already indicated the presence of an optically differentiated cell wall in some species (see Fig. 245), and will greatly clarify many of these obscure details in the near future. Cilia or flagella are present in some species, but their *modus operandi* is unknown. Some bacteria secrete around themselves a mucilaginous envelope often spoken

\* The micron ( $\mu$ ) is the unit of length used in bacteriology. It is  $\frac{1}{1000}$  of a millimeter or  $\frac{1}{25.400}$  of an inch.

of as a capsule (Figs. 243, 266 and 293). That, in brief, summarizes the more important knowledge of bacterial anatomy, unless spores be included as an anatomical feature. It is pitifully limited.

**Capsules.**—It is probable that the majority of bacteria form capsular substance under some circumstances although capsules have not been demonstrated in all species. Here again the electron microscope is proving itself an invaluable aid to science. In most species large, easily seen capsules seem to be called forth only by certain particular circumstances of environment such as growth in the animal body, or on certain media. Some, for example *Leuconostoc*, form their capsular material best in sugar vats or in milk. There is good reason to believe that capsules may be formed for protective purposes, in many cases at least. Capsules, for example, are often correlated with virulence of pathogenic forms such as pneumococci and the organisms of whooping cough, gonorrhea, meningitis, anthrax and other diseases. The capsules seem to enable these bacteria to evade the defensive mechanisms of the infected body. The bacteria often cease forming capsules when removed from the body to laboratory test-tube media.<sup>4</sup>

*The chemical composition of capsules* varies widely with species and they contain substances of great interest to the bacteriologist. Some are composed mainly of carbohydrates or gums, others contain nitrogen and phosphorus and may be of the nature of mucus or the mucins. Still others may be largely composed of inorganic gel-like substances. As will be emphasized in the discussion of streptococci and pneumococci, capsular substance has chemical properties which enable us to distinguish between various closely similar types of organisms which could not otherwise be differentiated.

*The relation of capsules to the cell* is not known definitely. In some cases they seem to be a thickened layer of the outer membrane; in others, a secreted mucoid substance not part of the cell but adhering to it. Some bacteria secrete a mucoid substance into their environment, but this may not be a true capsule and may bear no more true structural relation to the cell proper than any excreted waste product. A collection of organisms embedded in their secreted slimy substance is often referred to (improperly) as a *zooglea* or zooglear mass. The difference between zooglear material and true capsules is not entirely clear.

*Demonstration of Capsules.*—There are several methods of demonstrating the presence of capsules on bacteria. As the capsules differ so greatly in composition, however, they are not all amenable to

the same process. Three commonly used methods are described here.\*

**Flagella.**—The power of motion is obviously an asset to any living thing. In bacteria it appears, like the power of spore formation, to have developed only with elongation of form. With *one or two doubtful exceptions*, none of the spherical bacteria is motile, while many of the straight rod forms and most of the spiral forms can move about.

The motion of bacteria is usually accomplished by means of flagella arranged in various ways according to the species.<sup>5, 6</sup> Various arrangements of flagella are seen in Figure 42.

**\* India ink method for capsule demonstration:**

India ink which is relatively free from bacteria must be used. Often it is possible, by making examinations of several bottles of Higgins' "American India Ink," to find such material. (There are usually present, in India ink, certain cocci growing in slimy masses. Ordinarily these are easily distinguishable from pneumococci and streptococci by their large size, and arrangement in clumps.)

A small mass of the organisms to be examined is suspended in broth or water, and is then mixed with a drop of India ink and spread in a thin, gray film over the slide.

After drying, immerse the slide for 1 minute in pure methyl alcohol and then counterstain with safranin or methylene blue. The cell is colored, the background is granular and dark (ink), while the capsule is a colorless halo.

**Capsule stain (Churchman):**

An aqueous suspension is made from the 18-hour-old growth on an agar slant. This is smeared on a clean slide and air-dried.

The smear is covered with 10 drops of freshly filtered Wright's stain and this is left on till it is almost dry (3–6 minutes).

Wash with Clark and Lubs buffer solution (pH 6.4–6.5) and fan dry *without blotting*. (Sometimes rapid washing with distilled water after the buffer solution is advantageous.)

Under some circumstances the film may be soaked in Wright's stain overnight and then washed and air-dried.

**Capsule stain (Welch method [modified]):**

A. Crystal violet

Saturated alcoholic crystal violet . . . . . 5 cc.

Distilled water . . . . . 95 cc.

B. 10 percent  $\text{CuSO}_4$  . . . . . 1 liter

1. Make thin smear.

2. Dry in air.

3. Fix by gentle heat.

4. Cover with crystal violet solution.

5. Heat to steaming for 1 second. (Do not boil!)

6. Use no water.

7. Rinse *immediately* and *copiously* with copper sulfate solution

8. Use no water.

9. Blot dry *immediately*.

10. Examine.

The organisms are stained purple and the capsule appears as a faintly blue halo

Nothing definite is known of the mechanism by which bacterial cilia or flagella are moved, so that no discussion of this will be attempted. We merely know that many bacteria move, and that they usually do so by means of flagella.

Bacteria can move only in fluids. They cannot leave a fluid surface. They may pass through the air, but not of their own volition. It is only as passengers or riders on particles of dust in the air, as Spallanzani, Pasteur and Lister demonstrated, or on objects like

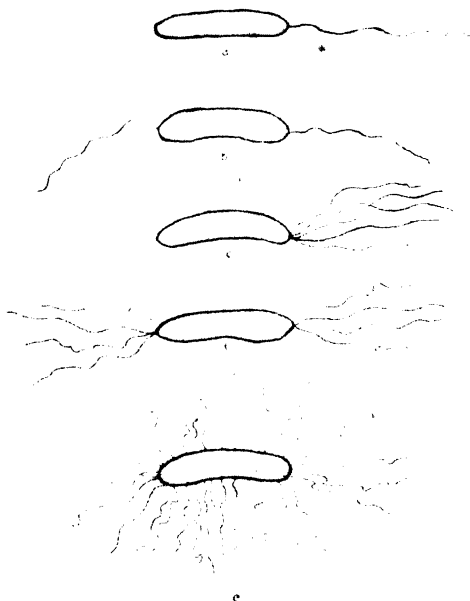


Fig. 42.—Types of flagellation: *a*, Monotrichate; *b*, amphitrichate; *c* and *d*, lophotrichate; *e*, peritrichate. (Redrawn from Topley and Wilson.)

pencils, eating utensils, books, etc., or in droplets such as those given off by a person when sneezing or coughing, or in streams of water, that they can travel great distances.

**Bacterial Motility.**—Bacterial motility is of a very primitive but useful type. The organisms apparently cannot control their speed, which seems always to be at a maximum for a given individual. Whether they can voluntarily change their direction of travel as do paramecia is open to question.

Their speed is sometimes very great when measured by their size.

Some of them cover a distance equal to hundreds of times their length in a second. An automobile would have to speed at a thousand miles an hour to accomplish the same thing.

*Demonstration of Motility.*—The *motility* due to flagella is easily visible upon direct observation of the bacteria in a droplet (hanging drop) of the fluid in which they are living. In any culture of bacteria, especially old cultures, motile cells may be difficult to find among thousands of dead or senescent cells. Young cultures should always be used. In cultures acidified by fermentation, bacteria lose their motility. Strict anaerobes lose motility on contact with air.

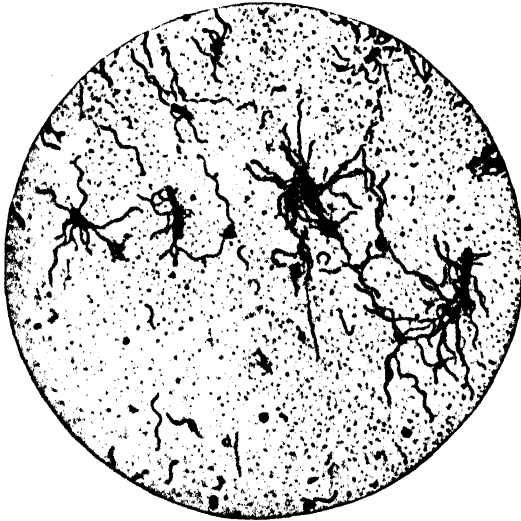


Fig. 43.—Typhoid bacilli, showing the wavy threadlike outgrowths (flagella) by which the bacilli move through fluids. The bacilli must be specially stained to make these outgrowths visible. (Williams.)

It is necessary to distinguish carefully between true motility and brownian movement. Truly motile bacteria progress definitely and continuously in a given direction. Brownian movement is a purposeless oscillation within a very limited area. It is due to molecular forces entirely external to the bacteria. Unless the bacteria are fairly active, brownian movement is sometimes rather difficult to distinguish from true motility.

*Demonstration of Flagella.*—The demonstration of bacterial flagella is not so easy as the demonstration of motility. In practice it is customary to regard motility as *prima facie* evidence of the pres-

ence of flagella. This is generally safe evidence when dealing with true bacteria (Eubacteriales) but in at least three other orders of the class Schizomycetes (Spirochaetales, Myxobacteriales, and Chlamydobacteriales) motility is accomplished without the aid of any visible means of locomotion and is not fully explained. Recent electron microscope observations by Mudd, Povitsky, and others show what had never previously been seen, flagella on spirochetes of syphilis (see Fig. 336).

Bacterial flagella easily become detached and are so fine as to be visible only when stained (Fig. 43). The staining of these organs is rather difficult and this may be regarded as evidence that they differ in some way from the body or *somatic* part of the organism, the latter being easily stained.<sup>7</sup> This point will be referred to again later. Useful means of staining flagella are those recommended by Gray\* and by Leifson.† It must be pointed out that students will

\* Gray's method:

I. Mordant:

Solution A

Potassium alum (sat. aq. sol.)	5.0 cc.
Tannic acid (20 percent aq. sol.)	2.0 cc.
(This must be preserved in the ice-box with a few drops of chloroform if a large amount is kept on hand.)	
HgCl <sub>2</sub> (sat. aq. sol.)	2.0 cc.

Solution B

Basic fuchsin (sat. alc. sol.)	0.4 cc.
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Mix solutions A and B only on the day they are to be used.

Separately they keep well, but deteriorate after mixing.

II. Stain:

Carbolfuchsin as prepared for Ziehl-Neelsen stain.

1. Flood smear with mordant and allow to act for 5 minutes. Rinse off gently.
2. Flood with carbolfuchsin for 5 minutes. Do not heat. Rinse off gently.
3. Blot dry.

† Leifson's method (Stain and mordant):

Ammonium alum (sat. aq.)	2.0 cc.
Tannic acid (20 percent aq.)	1.0 cc.
Distilled water	1.0 cc.
Alcohol (95 percent ethyl)	1.5 cc.
Basic fuchsin (sat. alc.)	0.3 cc.

1. Mix in the order given.
2. Flood the unfixed smear with this solution and allow to act for 10 minutes at room temperature, or until a metallic scum forms over the surface.
3. Wash in water.

Counterstain:

1. Flood with methylene blue 1 to 2 minutes
2. Wash in water.

ordinarily not find it as easy to accomplish the task of staining flagella as these directions make it appear. The factors involved are not well understood. However, a little perseverance is usually rewarded with success. To accomplish the best results the slides must be clean and free from grease and scratches, and the cultures must be carefully prepared. Some suggestions are as follows:

- (a) Place slides in cleaning solution overnight; wash; dry from alcohol, *or*
- (b) Cover with moistened Bon-Ami; dry; wipe with clean cloth.
- (c) *Moist* agar slant cultures 12 to 24 hours old are best, although broth cultures often show flagella well. In case one fails, try the other.
- (d) Make light emulsion in sterile distilled water and incubate 10 to 15 minutes to develop and extend the flagella fully.
- (e) Place large loopful on slide and allow to spread by tilting.
- (f) Do not *fix* by heat.

**Spores.**—Only two genera of true bacteria (*Bacillus*<sup>s</sup> and *Clostridium*) form spores, although sporelike bodies called conidia are produced by certain species of *Actinomyces* (see page 647). Both genera of spore-forming bacteria named above are rod forms, the former aerobic, the latter strictly anaerobic. When a rod is to sporulate, a tiny refractile granule appears in the cell. The granule enlarges and becomes glassy and transparent and resists the penetration of various substances. Disinfectants cannot readily penetrate into spores, nor can aniline dyes. A rod with a spore inside, when stained by ordinary methods, may thus appear to have a hole in it (Fig. 239a). However, the outer shell of the spore may readily be colored so that in stained smears spores may appear as tiny, blue rings or ovals (Fig. 44). All of the protoplasm of the rod seems to condense itself into the granule, or young spore, in a dehydrated, hard, resistant state. The empty cell membrane of the bacillus may separate off, like the hull of a seed, leaving the spore as a free, round or oval body.

*Function of Spores.*—Sporulation of bacteria obviously is not a reproductive function since most species reproduce perfectly without demonstrable sporulation. Further, in bacteria only one spore is produced by each cell and this spore produces no *new* individual. In this respect bacterial spores differ from the spores of true fungi and higher plants.

There is little or no metabolic activity in a spore, since all metabolism depends on a suitable state of hydration. Not until moisture is present do bacterial spores germinate. They represent merely a

dormant and resistant state which enables the bacteria producing them to survive during periods unfavorable to active vegetation.

When dry spores are placed in a suitable nutrient solution, the protoplasm within the shell imbibes water, swells, bursts forth and goes on as before.

*Cause of Sporulation.*—Just what induces a given cell to go into the dormant spore stage is unknown. Sporulation is not necessarily a response to unfavorable conditions, since spores are often formed early in the life of a culture while conditions are wholly favorable to continued vegetative activity. It may be that sporulation occurs at a certain age of the individual cell and represents a sort of resting and rejuvenating process, but this has not been shown to be the case.

*Resistance of Spores.*—When protein or protoplasm is moist or well hydrated, as is its condition in an actively growing, "vegetative," or live condition, it coagulates readily and is therefore vulnerable with respect to heat, chemicals, etc. The "white" of egg is a good illustration of a hydrated protein. It coagulates ("hard boils") readily enough.

When protein is dehydrated, it is no longer readily coagulable. It is attacked only with difficulty by various chemicals, and withstands high temperatures and other conditions which would soon destroy it were it allowed to become hydrated.

The resistance of bacterial spores to heat, chemical disinfectants, drought, cold, sunlight and other unfavorable conditions suggests strongly that the protoplasm inside them is in a dehydrated condition. In this way they can survive where the active, vegetative, or hydrated stage would soon perish. So resistant are some bacterial spores that they will live for as long as 20 years or more on dry splinters of wood, grow after treatment in strong disinfectant solutions, survive an hour or more of boiling, and remain unharmed by an hour or more in a hot oven.



Fig. 44.—Spores stained by Löffler's methylene blue. Note that the stain has penetrated only the outer shells of the spores, giving them the appearance of empty rings.



**Staining Spores.**—Although ordinarily resisting the penetration of dyes, spores may, nevertheless, be stained if proper methods are employed. A number of spore stains are available; two very simple methods by Alessandrini\* and by Schaeffer and Fulton† are extensively used.

It is sometimes difficult or impossible to demonstrate spores by means of the microscope, even when staining methods are employed. Resort must then be had to tests for resistance of the culture to heat. Vegetative cells are nearly always killed by exposure to 80° C. for 15 or 20 minutes and usually by temperatures as low as 60° to 70° C. If a culture survives exposure to 80° C. for 20 minutes it almost certainly contains spores.

**Granules.**—In many bacteria stained by ordinary methods, granules are readily seen (Fig. 45). They are distinguished by the fact that they stain more intensely than the rest of the cell, or are of a different color. They are often spoken of as *metachromatic granules*.

The composition and function of these granules is, for the greater part, a matter of debate. As shown by their reaction to various test reagents, such as iodine, some of them are of starch (blue), glycogen (brown) or closely related compounds and doubtless represent reserve food substances since they tend to disappear during periods of food scarcity. In some species pure elemental sulfur serves the same purpose. This may be demonstrated by smearing the bacteria on a slide, and treating with carbon disulfide. After evaporation of

\* **Alessandrini's method:**

A. Ziehl-Neelsen's carbolfuchsin (as used for acid-fast stain).

B. *Fresh*, 5 percent aqueous sodium sulfite.

C. Methylene blue.

1. Make smear and fix in flame (do not overheat!).
2. Flood with carbolfuchsin and steam gently (do not boil).
3. Cool, with stain, 15 minutes. Wash in water.
4. Decolorize with sodium sulfite. Wash in water.
5. Counterstain with methylene blue, 1 minute. Wash in water and blot dry.

Spores stain red, bodies of bacilli blue.

† **Schaeffer and Fulton method** (modified):

A. Five percent aqueous malachite green.

B. Five percent aqueous eosin.

1. Flood smear with malachite green solution.
2. Heat over steam bath for 5 minutes (do not boil).
3. Wash gently with water.
4. Flood smear with eosin solution.
5. Rinse and blot dry.

Spores green, bacillary bodies red.

the disulfide the sulfur remains in characteristic crystal forms. In many bacteria, granules of a nitrogenous compound, *volutin*, are commonly observed. It is related to, but not identical with, the protein of the nucleus (nucleoprotein), being dissolved in hot water (80° C.) instead of coagulated by it, and not being digested by trypsin. It probably serves as a reserve of nitrogenous food.

Other granules have been observed from time to time and various interpretations of them have been offered. Some regard them merely as accumulations of waste products or degenerated protein in dying or dead cells; others ascribe reproductive functions to them or describe them as nuclear structures. Some of these ideas will be taken up again farther on (see page 74).

**Staining Granules.**—There are many methods of bringing granules into prominence, the method adopted depending largely on the chemical nature of the granules themselves. One commonly used to demonstrate volutin granules is that of Albert.\* Volutin granules are frequent in gram-positive bacteria.

**Fat Granules.**—Some bacteria synthesize and store much fat, especially if fed with sugar or other fermentable food—in this show-



Fig. 45.—Various kinds of bacteria with examples of granules. Observe that, in some, the granules appear to be on the outside of the cell.

\* Albert's method of staining volutin granules:

A. Toluidin blue .....	0.15 gm.
Methyl green .....	0.20 gm.
Acetic acid (glacial) .....	1.00 cc.
Alcohol (95 percent) .....	2.00 cc.
Water .....	100.00 cc.

Allow to stand 24 hours. Filter through paper.

B. Iodine as in Gram's stain.

1. Prepare smear as usual.

2. Flood with solution A for 1 minute. Wash with water and blot dry.

3. Stain with solution B for 1 minute, wash and blot dry.

Granules black; other parts dark or light green.

ing a striking resemblance to many human beings.<sup>9</sup> Some species of the genus *Bacillus* are outstanding in this respect, notably, *B. cereus* and *B. megatherium*. A 24-hour culture on glycerol agar, if properly stained (by osmic acid or sudan III), will show large amounts of fat. The method has been relatively little studied and offers a good means of differentiation between species.\*

**The Nucleus of Bacteria.**—No discussion of a living cell is complete without some consideration of the nucleus. Unlike the protozoan cell, or the cells of more highly organized plants and animals, bacterial cells possess no definite, clear-cut and well-differentiated nuclear structure. The nuclear material of all bacteria known today consists, in all probability, of minute chromatin† granules scattered diffusely throughout the cell in an arrangement less highly organized even than the primitive diffuse nuclei in the blue-green algae.<sup>10</sup>

It has been suggested that bacteria are so primitive that a nucleus is unnecessary to their existence. As a matter of fact, the primitiveness of bacteria is only an assumption and not a proven fact, so that this idea is not well supported. Further, it is unlikely that such stable and durable creatures should live without the one structural unit which is believed necessary to propagation and heredity of all types of cells. In addition, bacteria stain in a manner which suggests that, far from containing no nucleus, they may consist almost entirely of nuclear material, cytoplasm being absent or present in very small amounts.

Many of the granules referred to in the preceding section have been believed to represent nuclei or nucleus-like collections of proto-

\* *Fat stain* (Burdon, Stokes and Kimbrough)\*

1. Prepare a saturated solution of Sudan Black (Nat. An. and Chem. Co.) (0.4 percent) in 70 percent alcohol in a stoppered bottle. Stand at room temperature at least 24 hours. The solution is good for at least a month. Filter through paper just before use and put 0.5 cc. of the filtrate into a small tube for each culture to be stained.

2. Emulsify growth from a 24-hour glycerol-agar slant directly in the solution. Allow to settle for 20 minutes.

3. Smear a loopful from the top of the fluid on a slide. Dry without heating.

4. Counterstain for 10 to 15 seconds with safranin (1 per cent aqueous), rinse gently, dry and examine.

Fat is bluish-black or gray, cytoplasm pink.

† *Chromatin* is a form of protoplasm believed to represent the most vital and fundamentally essential part of living matter and it is of chromatin that the very earliest and most primitive living beings are thought, by some authors, to have consisted. It is named from the fact that it has a marked affinity for certain coal-tar dyes. The nucleus, the most primitive and essential part of all cells, is composed of chromatin and stains most deeply.

plasm, but no convincing evidence in support of this view has been forthcoming. It would seem not unlikely that nuclear material may undergo temporary local concentrations in certain parts of the cell under some circumstances, but these would not necessarily constitute nuclei.

**Cell Membrane.**—We have already learned that the living bacterium consists largely of protoplasm. The physical and chemical interrelationships of the colloidal materials composing protoplasm are extremely delicately balanced. Since these colloidal materials are enclosed within a membrane of some sort, which not only prevents their dissolution and protects them from environmental influences, but selects their food and excretes their wastes, it will be of help, in understanding certain relationships of bacteria to their environment, to give a few minutes' consideration to this important part of the bacterial cell.<sup>11</sup>

The cell membrane differs in various classes of cells, having sometimes a definite structure, in other instances seeming to consist merely of a somewhat condensed layer of outer cytoplasm. It may have various chemical compositions such as chitin or cellulose, or various substances such as mucin, hemicellulose, gums and related compounds, depending on the species of organism.

Whatever the composition of the cell wall or membrane, it is, in the case of bacteria, the medium through which must pass not only all nutrient material for the cell, but also all waste matter, enzymes and other products given off by the cell during its life. The membrane is *permeable* to these substances, but *impermeable* to others and is therefore said to be a *semipermeable membrane*. In order to retain within the cell the proper substances in the proper proportions, and to prevent the entrance and allow the escape of improper or waste substances, it must exert a very fine discrimination or selective permeability at all times.

Any slight defect or alteration in the discriminatory properties of the cell membrane must, therefore, have a profound effect on the cell contents. When, as is the case with bacteria, the entire individual consists of the cell contents and depends for its existence as a living entity on the chemical and physical equilibrium of its protoplasm, then the maintenance of the integrity and uniformity of the cell membrane is a matter of the greatest moment. Substances or forces which tend to alter the membrane or overcome its selective powers must influence the well-being of the cell.

**Osmotic Pressure.**—The student will doubtless have learned in his courses in biology and physics that if a semipermeable membrane

like fish-bladder separates two solutions, one having a high salt or sugar concentration and the other a low one, water will tend to pass (diffuse) through the membrane toward the solution of higher concentration till the concentration is in a state of osmotic equilibrium on both sides. The solution of higher concentration is said to have a higher *osmotic pressure* than the other. If living cells are immersed in fluids having extremely high or low osmotic pressures, the normal selective permeability of the cell membrane may be overwhelmed, for a time at least.

Well-known examples of the effects of unfavorable osmotic pressures are found in the plasmolysis and plasmoptysis seen in cells of certain plants when they are immersed in solutions having too high (hypertonic) or too low (hypotonic) a salt concentration. Likewise, if we immerse certain bacteria, especially delicate parasitic forms, in a solution of very high osmotic pressure, water is probably drawn from them and they may be injured or die before equilibrium can be established. If they are immersed in distilled water the reverse process may occur with deleterious effects. It is for this reason that the osmotic pressure of fluids used for the cultivation of bacteria is adjusted by the addition of measured quantities of salt or other substances.

*Effects of Evaporation.*—When a solution containing salt or other soluble compounds is exposed to the air so that evaporation occurs *slowly*, the water, departing, leaves behind a more and more concentrated solution, the osmotic pressure becoming greater and greater. Bacteria suspended in such a fluid are unfavorably influenced by the increasing osmotic tension. In the cultivation of bacteria, therefore, evaporation of moisture from the medium is to be avoided. This may be accomplished by maintaining a very humid atmosphere in the incubator.

*Alterations in Membrane Permeability. Involution Forms.*—Even though the osmotic pressure of a given culture medium may be appropriate, there often appear in it substances that alter the permeability of the cell membrane in some way so that the cells become swollen and distorted. Such alterative substances may be the waste products (acids, alcohol, etc.) of bacterial cells which have grown in the culture. The cell membrane may also become altered through aging so that it fails to function properly, very likely permitting the passage outward of important cell constituents and the passage inward of water or of deleterious compounds and probably causing the swellings, knoblike protrusions and other irregularities often observed in old cultures.

These swollen and distorted forms of bacteria have been observed by bacteriologists for many years and generally are spoken of as "involution forms." In certain species (*e.g.*, *V. comma*) such distortions may be brought about to a very striking extent by cultivation of the organisms on media containing high concentrations of sodium chloride (Fig. 46). Many modern students of the subject, however, attach importance to some of the irregular forms of bacteria as representing stages in life cycles and complicated reproductive processes. The whole question is an open one and those who have time and inclination to investigate it will find much of interest and value.

### Chemical Composition of Bacteria.

—In spite of their supposedly primitive nature, bacteria are not markedly different in chemical composition from other living cells. However, the exact chemical analysis of *living* matter is difficult, if not impossible, since to analyze it one must kill it and disturb the interrelations of various components of the cell. Curran and Brunstetter have made interesting comparisons of chemical composition of spores of bacteria with that of vegetative cells by means of spectro-chemical analysis. Increased calcium seems to be related to increased heat resistance.<sup>12</sup> Such chemical data as are available for bacteria correspond, in a general way, with data for other plant and animal cells. Thus, bacterial protoplasm is composed chiefly of the elements C, O, H, S, N and P, with smaller amounts of many other elements as Na, K, Ca, Fe, Mg, Cl and so on. It is known that the ratio of the last group of elements and some of the first may vary with the composition of the medium on which the bacteria are growing at the time they are analyzed. This is true in a marked degree of all other constituents of the bacterial cell.

Like other protoplasm, water is an important component, con-



Fig. 46.—Involution forms of *V. comma* (cholera vibrio) when cultivated upon agar containing excessive amounts of salt. (Compare with Fig 236.)

stituting roughly from 75 to 98 percent of the whole weight. The ash may vary in amount from 2 to 30 gm. percent of dry weight of bacteria; and protein substance including globulins, nucleoproteins, etc., from 12 to 90 percent of the dry weight. Many complex, non-protein, nitrogenous substances are also found, among them being various purine bodies, polypeptides, amines and amino acids.

The carbohydrate content is also very variable since, as has been pointed out, many bacteria store up granules of polysaccharides for later use. Figures such as 12 to 35 percent of total dry weight are found in various species and various analyses. Such carbohydrates as cellulose, glycogen, starch, gums, dextrose, etc., have been reported, as well as more complex polysaccharides in the capsules of some species. In the order Thiobacteriales sulfur (stored in elemental form) is an important constituent of the cells.

In some genera, as that (*Mycobacterium*) containing acid-fast organisms like the tuberculosis bacillus, waxes are an important component, while fats and other lipoids are present in nearly all cells to some degree.

*Pigments* are a prominent feature in many species of bacteria. Their exact function is not known, nor is their chemistry, except in a general way. Chemically, the pigments may be grouped as:

- (a) *Carotinoid*—lipoids of red, yellow and orange shades.
- (b) *Melanins*—soluble in strong acids and alkalis; black and brown.
- (c) *Anthocyan*s—water- and alcohol-soluble pigments of red, blue and violet tints. Also greens and yellow colors.

The anthocyan may appear as colorless compounds called leuco-bases when first formed by the bacteria, becoming colored only on oxidation.

Physiologically, with respect to pigments bacteria may be roughly grouped under four headings:

- (a) *Chromophoric*—pigment is held in the cytoplasm in a loose, chemicophysical combination (Staphylococcus; Actinomyces).

In the order Thiobacteriales there are pigments belonging to the photosynthetic group, like chlorophyll. These are magnesium-bearing compounds and their function of photosynthesis has been clearly demonstrated by van Niel<sup>13</sup> and others.

- (b) *Para-chromophoric*—pigment is intracellular, but not in the cytoplasm as a compound (*Torula*).
- (c) *Chromoparous*—excrete the pigment into the surrounding medium (*Serratia*).
- (d) *A-chromoparous*—pigment is excreted in the form of a leuco-base (*Pseudomonas aeruginosa*).

**Bacterial Pigments.**—Bacterial pigments in general vary greatly in amount and intensity of color, even in the same organism.

Growth at various temperatures and on various media affects either the mechanism responsible for pigment formation or the color of the pigment itself. Anaerobic growth usually results in lack of, or discoloration of, bacterial pigment. A pigmented species may give off nonpigmented variants.

Chemically, bacterial pigments are of diverse natures, as judged by the fact that some are soluble in water, others in alcohol and some in neither. Actually, little is known of their exact composition.

In general, pigments may be intracellular, or they may pass through the semipermeable membrane of the cell and so be extracellular. *Prodigiosin*, the pigment of *Serratia marcescens*, is of the extracellular type (see page 427).

*Function of Pigments.*—The physiological significance of bacterial pigments is an interesting problem in view of the role of chlorophyll in the life of plants containing it. There seems to be insufficient evidence to support the view, held by some, that pigments protect bacteria from light. With the possible exception of the blue pigment pyocyanin of *Pseudomonas aeruginosa*, and one other mentioned below, they do not play a part in respiratory processes or in any synthetic process such as that carried on by chlorophyll. Bacterial pigments may be merely products of metabolism, just as urea and carbon dioxide are products of human metabolism, although the chemical processes involved in the formation of these three compounds are entirely different. That there is a definite relationship between light and pigment formation is clear. Baker<sup>1</sup> showed that dead bacteria do not become pigmented on exposure to light, nor do alcoholic extracts of pigments. Ultraviolet and sunlight irradiation induced bacteria to produce pigment when subsequently growing in the dark. Many bacteria would not produce pigment in the dark, but did so when growing in the light.

An exception to above statements is found in the green bacteriochlorophyll of the autotrophic, alga-like sulfur bacteria (Thiobacteriales). The pigment in these species is intracellular and has a function analogous to that of chlorophyll (see section on Thiobacteriales, page 395).

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around 70° C. for 1 to 15 minutes. Boiling kills them in a few moments. Spores are much more resistant, as has already been pointed out.

The relations of temperature to the *growth* of bacteria are, however, somewhat more complex. Many species in the soil, water, air and body grow well at temperatures from 25° to 40° C. (human body temperature is 37° C.). Species growing well at such temperatures are called *mesophilic* (moderation-loving). Some of the soil and water bacteria grow best at temperatures very little above the freezing point (4° to 10° C.) and these are referred to as *psychrophilic* (cold-loving). Certain marine forms, adapted to life at around 4° C., will die if held above about 30° C. for more than a few minutes.<sup>2</sup> These would be called *obligate psychrophils*, i.e., they are limited to this temperature as a condition of life. There are, in addition, several species of bacteria which thrive only at high temperatures (60° to 80° C.). Such species are called *thermophilic* (heat-loving). Some species of thermophilic bacteria are found in hot sulfur springs while others occur in milk and soil. A distinction should be made in all cases between ability to *endure* a given temperature and ability to *grow well* under the same conditions. Many bacteria, for example, can *live* for months at freezing, yet do not *grow* at all.

The bacteria which cause disease in warm-blooded animals are adapted to grow best at body temperature (37° C.) and medical bacteriology, therefore, deals chiefly with mesophilic bacteria. Most of the bacteria active in the "spoiling" of vegetables, the decay of meat and the souring of milk are also mesophilic and grow very slowly, if at all, at refrigerator temperatures (4° to 10° C.) For this reason, foods may be preserved for days or weeks if properly refrigerated. Refrigerators should be frequently cleaned and aired to discourage psychrophilic molds and bacteria.

*Extreme Cold.*—Many species of bacteria are highly resistant to extremes of cold, even when in the vegetative state. In this respect they are probably unique among living things and it may be due to this that certain species of bacteria may have survived glacial epochs in the earth's history. Many species, even parasites like the typhoid organism, will survive for weeks frozen in ice. Turner has maintained syphilis spirochetes frozen in "dry ice" at -78° C. for years without loss of infectivity.<sup>3</sup> Some species of bacteria will grow, apparently unaffected, even after subjection to the temperatures of liquid air. Larger cells than bacteria are often entirely disrupted by the crystallization (freezing) of their fluids. In the use of low tem-

peratures freezing should be *very rapid* (and thawing equally fast) to avoid the formation of large ice crystals.

**Thermal Death Point.**—Some non-spore-forming bacteria are definitely more heat-resistant than others. Thus, the non-spore-forming varieties of thermophils may thrive at 60° or 65° C., which soon kills some species, especially pathogenic varieties. These may not survive at 50° C.

It is often necessary to measure the heat resistance of bacteria accurately, and various phrases have been coined to express the results of such measurements. For example, the temperature at which all the bacteria of a given species are killed after 10 minutes' exposure is spoken of as the "thermal death point" of that species. This is a very inexact expression, however, because studies have shown that at a given temperature, say 70° C., the bacteria do not all die simultaneously and suddenly just as the clock registers the expiration of 10 minutes. "Thermal death point" merely tells us when the *last survivor* of all has expired, the number of live cells having been decreasing continuously during the exposure period. In measuring thermal death point much depends on the species and numbers of bacteria originally present, the age of the bacteria, the acidity of the suspending fluid, its osmotic pressure and its composition, etc., so that the term "thermal death point" can be correctly used only if the exact conditions of a given experiment are known. However, for certain purposes, the information is of practical use.

**Thermal Death Time.**—This is the length of time required to kill all of the bacteria in a given substance at a stated temperature. The determination of thermal death time is subject to much the same errors as the measurement of thermal death point, but under known conditions is of practical use. Thus, in pasteurizing, milk is heated to 62° C. and held there for 30 minutes. Experiments have shown that this is considerably in excess of the *thermal death time*, at 62° C., in *market milk*, of all the disease bacteria known to be transmitted by milk. Many non-pathogenic bacteria, both spore-forming and non-spore-forming, survive pasteurization so that pasteurized milk is safe but not necessarily sterile.

**Rate of Death.**—As noted in the section on *thermal death point* (q.v.), death of all the bacteria in a given material does not occur simultaneously unless the lethal influence be overwhelming. On the contrary, death occurs in a definite relationship to time, the rate being determined by various factors such as temperature, moisture, pH, etc. The process behaves like a monomolecular chemical reac-

tion, progressing in accord with the laws governing mass action. If the numbers of organisms surviving in a disinfection test be determined at various intervals, and plotted against the intervals, it will be seen that regular curves are formed. If the logarithms of the numbers be similarly plotted, a straight line is formed (Fig. 47).

**Hydrogen Ion Concentration.**—Another physical factor profoundly affecting bacteria is the concentration of acid or alkali in the fluid in which they are suspended. This is usually expressed in terms of *hydrogen ion concentration* since, as the student will recall

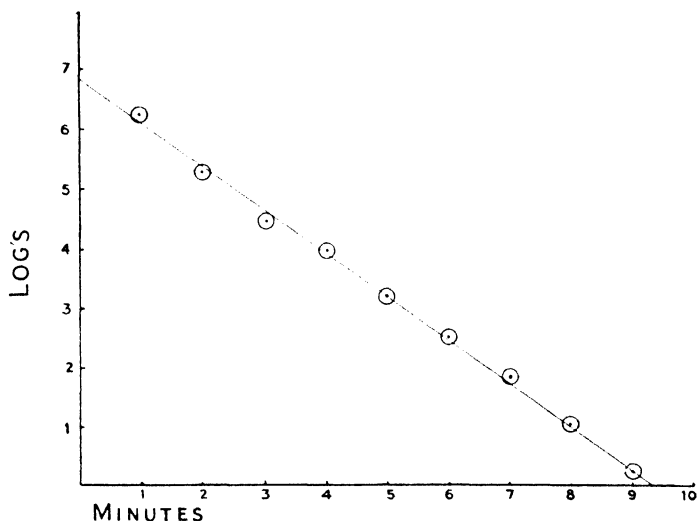


Fig. 47.—Relation between numbers of organisms and time in a 1 percent solution of phenol containing typhoid bacilli. The logarithms are of numbers of bacilli per cc. The straight-line curve is characteristic of the relation between time and survivors under any definitely adverse conditions when no growth occurs.

from his studies of chemistry and physics, it is the concentration only of *dissociated* or *ionized* hydrogen (or hydroxyl) that determines the actual, *effective acidity* (or alkalinity) of a given solution.<sup>4</sup> Unfavorable influences of many sorts may be much enhanced in fluids having an acid reaction. For example, coagulation by heat occurs more readily in acid solutions. Thus, milk which is only very slightly sour may curdle on being warmed.

The reaction of bacteriological culture media must therefore be very carefully adjusted, with respect to *hydrogen ion concentration*, the degree of acidity depending on the bacteria to be cultivated.

For example, bacteria parasitic in the human body are adapted to an environment having a slight alkalinity, since the reaction of the blood is slightly alkaline. Many other bacteria (*e.g.*, *Azotobacter*) also prefer slightly alkaline media, but there are numbers of species which thrive best in a slightly acid environment (*e.g.*, *Lactobacillus*, *Brucella*). It is necessary, therefore, to adjust the reaction of any culture medium so that it approaches that at which the bacteria we desire to cultivate will grow best. A method by which this is done is described in Chapter 7.

**Titration Acidity and Hydrogen Ion Concentration.**—Students familiar with the theory of electrolytic dissociation will remember that strong acids are those which, when dissolved in water, dissociate largely into positively charged hydrogen ions and a negatively charged ion, which is often a radicle. For example, each molecule of sulfuric acid dissociates into 2 H ions and a sulfate ion or radicle. Weak acids like acetic or citric also dissociate, but to a much lesser degree. The student will remember that the acidic *activity* of any acid solution depends upon the concentration of these ions of hydrogen and this is obviously dependent upon the ability of the acid to give them off into the solution or to dissociate. Thus, two acid solutions may be of the same total concentration with respect to the *total* amounts of hydrogen available, yet have widely differing activities due to differences in the amount of active or *ionized* or *dissociated* hydrogen. Here we deal with a *capacity* effect, *i.e.*, total available (dissociated plus undissociated) acid, and an *intensity* or activity effect (dissociated acid or H ions alone).

As an example, let us consider acetic acid (vinegar acid) and hydrochloric acid. A liter of a normal solution of each contains exactly 1 gm. of total *available* hydrogen, yet the *activity* of the N/1 acetic acid is slight while that of the N/1 hydrochloric acid is great. Of the gram of available hydrogen in the acetic acid solution only about 1.36 percent is in an ionized state, so that there are, in the liter of solution, only 0.0136 gm. of H ions.\* The gram of hydrogen in the liter of N/1 HCl solution is about 91.4 percent ionized, giving 0.914 gm. of H ions per liter.† The N/1 HCl, therefore, is about 67 times as active or “strong” as the N/1 acetic acid.

If one were to titrate the solutions, *i.e.*, add N/1 NaOH solution until each became neutral, the *total* amount of alkali required would be exactly the same in each case. This is due to the fact that, as the alkali combines with the H ions, more H ions take their place from the undissociated acid which strives to maintain a constant H ion concentration consistent with its dissociation constant. Each acid finally gives up all its available hydrogen and, since each of the solutions by definition (N/1) contained exactly 1 gm. of available hydrogen to start with, each requires the same amount of alkali for its neutralization.

A measurement of hydrogen ion concentration, therefore, differs from a titration, in that the former determines only the actual concentration of *ionized* or *active* hydrogen at the moment without calling out any of the reserve, undissociated acid; while the latter measures the total, available, *ionizable* as well as *ionized* hydrogen, and calls upon all the reserve, undissociated acid until it is exhausted and the solution has no acidic property left, or is neutral.

Very few acids dissociate so completely that their N/1 solutions contain 1 com-

\* Or  $0.0136N = N/73.5$ ;  $\text{Log } 1/73.5 = -1.84 = \text{pH } 1.84$ .

† Or  $0.914N = N/1.094$ ;  $\text{Log } 1/1.094 = -0.1$  or  $\text{pH } 0.1$ .

plete gram of ionized hydrogen per liter. The term "normal" generally refers to the presence of 1 gm. of total available hydrogen per liter of solution. It cannot, therefore, be properly used in speaking of total active hydrogen (H ions) unless the phrase "with respect to H ion concentration" be appended. Thus, a solution normal *with respect to H ion concentration* contains 1 gm. of H ions per liter, while a tenth normal (N/10) solution (*with respect to H ions*) contains 0.1 gm. of H ions per liter, and so on (see Table 1).

*Method of Expressing Hydrogen Ion Concentration.*—Pure water dissociates, ever so slightly, into H and OH ions. For each H ion there must obviously be one OH ion ( $\text{H}_2\text{O} \rightarrow \text{H}^+\cdot\text{OH}^-$ ). Therefore, the two kinds of ion are always present in equal amounts in pure water and the reaction of the "solution" is neutral. According to the laws governing this relationship, the ratio of the product of the ions, to the unionized water, is constant. Therefore,

$$\frac{(\text{H}^+) \cdot (\text{OH}^-)}{\text{H} \cdot \text{OH}} = K.$$

The quantity of  $\text{H} \cdot \text{OH}$  is so large in proportion to the ionized part that the ratio may be written:

$$(\text{H}^+) \cdot (\text{OH}^-) = K.$$

By conductivity experiments the actual concentration of H and OH ions in 1 liter of pure water has been found to be 0.000,000,000,001 ( $10^{-14}$ ) gram-ions. Since, in pure (neutral) water, the concentrations of H and OH are equal, then the concentration of H ions must be 0.000,000,1 ( $10^{-7}$ ) gram-ions. Instead of using complicated fractions and phrases such as "0.000,000,1 gram-ion of H," in order to express neutrality, a simpler system of numbers is used. The symbol *pH* is used with these numbers in place of the phrase "hydrogen ion concentration." Thus, in this system, the neutral point is expressed by the symbol *pH* 7. This numeral represents the logarithm of the fraction (0.000,000,1 or  $10^{-7}$ ) given above, expressed as a positive number.

Since the number representing *pH* is derived from the logarithm of a fraction, it is obvious that the larger the fraction the smaller will be the number in its logarithm. Therefore, numbers below 7 represent greater hydrogen ion concentrations or acidities, while numbers above 7 represent lower hydrogen ion concentrations or alkalinities. Table I shows some of these relationships.

**Buffers and Buffer Action.**—In the ordinary titration of, let us say, N/10 acid with N/10 alkali, in distilled water using phenol red as indicator, there comes a point in the titration when a single drop of N/10 alkali from the burette changes the reaction from

TABLE I

RELATIONSHIPS OF HYDROGEN ION CONCENTRATIONS EXPRESSED IN VARIOUS WAYS

Reaction	Fraction of Normality*	Hydrogen Ions per Liter†	Logarithms of H Ion Concentrations	Expressed as pH
Acid .....	N/1	1.0	— 0	0.0
Acid .....	N/10	0.1	— 1	1.0
Acid .....	N/100	0.01	— 2	2.0
Acid .....	N/1,000	0.001	— 3	3.0
Acid .....	N/10,000	0.000,1	— 4	4.0
Acid .....	N/100,000	0.000,01	— 5	5.0
Acid .....	N/1,000,000	0.000,001	— 6	6.0
Neutral .....	Pure water	0.000,000,1	— 7	7.0
Alkaline .....	N/1,000,000	0.000,000,01	— 8	8.0
Alkaline .....	N/100,000	0.000,000,001	— 9	9.0
Alkaline .....	N/10,000	0.000,000,000,1	— 10	10.0
Alkaline .....	N/1,000	0.000,000,000,01	— 11	11.0
Alkaline .....	N/100	0.000,000,000,001	— 12	12.0
Alkaline .....	N/10	0.000,000,000,000,1	— 13	13.0
Alkaline .....	N/1	0.000,000,000,000,01	— 14	14.0

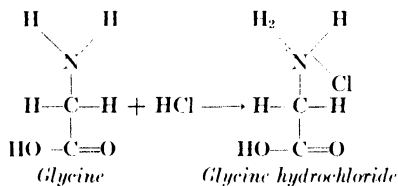
\* With respect to H or OH ions.

† Grams.

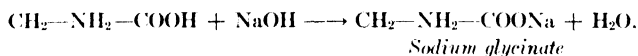
distinctly acid (yellow; pH around 6.5) to definitely alkaline (pH around 8 or 9) as evidenced by the appearance of a deep magenta color. Thus a single drop of N/10 alkali (or acid) produces a very large alteration in pH. In attempting an adjustment of the pH of bacteriological media such as infusion broth it is found that, unlike the titration of the aqueous solutions of acid or alkali, *no sharp end-point is reached*, and that the change from an acid to an alkaline reaction or vice versa is very gradual, requiring the continuous addition of relatively large amounts of acid or alkali. In other words, even at or near the neutral point, the solution being titrated shows a marked tendency to resist any change in its reaction. This is due to what is known as "buffer action" on the part of certain constituents of the broth. These substances combine with the acid or alkali as fast as it is added and so prevent change of reaction of the medium until they are all used up.

There are several sorts of substance which have the property of acting as buffers. Important among these, from a biological standpoint, are amino acids and their compounds, the polypeptides, proteoses, proteins, etc. These are neither strongly acid nor alkaline but will combine with either acid or alkali.<sup>5</sup>

To cite a simple example of buffer action by an amino acid, we may note that glycine combines with HCl or NaOH as follows:



or,

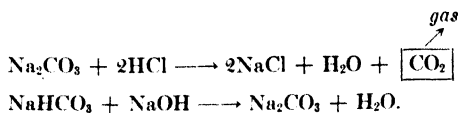


Since the hydrochloride or sodium salt of glycine is *very little dissociated*, little acidity or alkalinity of the solution results from their formation; hence, the *pH* tends to remain unchanged in spite of the addition of either acid or alkali.

Other important buffers are salts of weak acids, as acetates or phosphates. A mixture of sodium acetate and acetic acid is a good buffer mixture, since the acetate dissociates strongly and the acid weakly. If HCl is added, the  $\text{H}^+$  from the HCl and from the acetic acid together exceed the dissociation constant of both acids and enough of each acid reassociates to reduce the amount of free hydrogen ions to the initial *pH*. If this reassociation of the acids is not enough, then the excess  $\text{H}^+$  from the HCl combine with acetate ions of the Na acetate, forming NaCl, Na acetate and undissociated acetic acid. A reverse process occurs on the addition of alkali such as NaOH. Sodium acetate and water are formed by reaction with the acetic acid and, if this still leaves free  $\text{OH}^-$ , undissociated acetic acid will dissociate to liberate  $\text{H}^+$  which combine with the  $\text{OH}^-$ , thus maintaining the initial *pH* until all the acetic acid is used up.

One of the most commonly used buffers is a mixture of the monobasic and dibasic phosphates,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$ . When acid is added the dibasic salt absorbs  $\text{H}^+$ , changing into the monobasic and forming a K salt with the acid. When alkali is added, the monobasic salt gives up  $\text{H}^+$  to form  $\text{H}_2\text{O}$  with the  $\text{OH}^-$  radicle, and the incomplete salt combines with the metallic radicle of the alkali.

Other important buffer constituents of organic media are carbonates and bicarbonates:





Lumps of marble ( $\text{CaCO}_3$ ) are often added to cultures to maintain the  $p\text{H}$  unchanged, that is, to neutralize the acid produced by fermentation. Colloidal substances such as charcoal, precipitates of phosphates, kaolin, etc., which adsorb  $\text{H}$  or  $\text{OH}$  ions directly, are also active buffers and are used in biological systems.

**Ions other than  $\text{H}^+$ .**—Salts of various acids and metals sometimes produce marked biological effects in relatively minute amounts.<sup>6</sup> The favorable or unfavorable action of any salt such as  $\text{KCl}$ ,  $\text{Na citrate}$  or  $\text{HgCl}_2$ , depends on a number of factors among which are the ions released by the salt,  $p\text{H}$ , temperature, presence of organic matter, presence of other salts, kind of organism, and the like.

In respect to unfavorable effect, salts of the heavy metals in general are more toxic to bacteria than those of the light metals. Monovalent metals are usually less toxic than multivalent metals. The toxicity of most salts is reduced by the presence of protein, which reduces the various ion concentrations by physico-chemical combinations. Other things being equal we may say that chlorides of the alkaline earth metals ( $\text{Na}$ ,  $\text{K}$ ,  $\text{Li}$ ,  $\text{Sr}$ ,  $\text{Mg}$ ,  $\text{Ca}$ ) are relatively harmless, and even desirable and stimulating to most bacteria in the order named, with  $\text{KCl}$  and  $\text{NaCl}$  usually harmless in concentrations as high as 1.5 percent and not exerting very striking effects on many bacteria, after adaptation to artificial cultivation, even in concentrations of 4 or 5 percent. In general, chlorides of the heavy metals ( $\text{Zn}$ ,  $\text{Cu}$ ,  $\text{Fe}$ ,  $\text{Pb}$ ,  $\text{Hg}$ , etc.), while slightly stimulating in *extremely* low concentrations (*e.g.*,  $\text{Hg}$  : 0.000.005M), exert definitely harmful effects if present in greater amounts than about 0.007 percent in the case of mercury, or 0.008 percent when the metal is lead.

Salts act favorably in a great variety of ways, sometimes by effectively suppressing the ionization of unfavorable substances, or by reacting with these substances so as to prevent them from affecting bacteria unfavorably. For example, the presence of calcium chloride might be very effective in reducing the toxicity of sodium oxalate by forming an insoluble precipitate of calcium oxalate, thus removing the toxic oxalate radicle from the solution. Salts probably act also by altering the permeability of the cell membrane and by affecting its electrical charge, enzymes, etc. The importance of this action in metabolism and immunology is discussed in the chapters on those topics. Salts also act by forming chemical compounds with constituents of the cell substances; whether favorable or unfavorable compounds depends on the salt and conditions.

**Influence of Anion.**—In addition to the metal (cation) of which a salt is composed, its desirability or toxicity from the standpoint of

bacteria is determined in great part by the acid radicle (anion) with which it is combined. Most of the experiments on salt effects have been made with *Escherichia coli* as a test organism. In order of favorable action with regard to bacteria (especially *E. coli*) we may list several anions as follows: sulfate, tartrate, chloride, nitrate, acetate, citrate, oxalate, iodide, benzoate, tellurate; sulfates the least toxic, tellurates the most unfavorable. There are many others intermediate between these. Sodium chloride is a highly desirable component of nutrient media. Sodium benzoate is strongly bacteriostatic or bactericidal, and is an effective food preservative in concentrations as low as 0.1 percent. It is widely used commercially for this purpose. Benzoates in general are unfavorable to bacteria. On the other hand, the sulfate radicle is highly toxic when associated with copper, but is one of the least toxic of ions when associated with sodium or potassium.

The same general relationship extends to acids and alkalis. For example, hydrochloric acid and sulfuric acid, although "strong" acids, are much less poisonous to most bacteria at a given pH than benzoic acid or acetic acid; about 7.5 to 7.7 parts per million of the former two are required to produce the same toxic effect as 0.1 and 1.2 parts per million, respectively, of the latter two. Barium hydroxide, a much weaker (less dissociated) alkali than sodium hydroxide, is nevertheless much more toxic at the same concentration of hydroxyl ions. This is because the barium ion is more toxic than the sodium ion.

These generalizations must be modified to cover differences between various species of bacteria. For example, *C. diphtheriae* grows well in the presence of tellurites, which are toxic to many species, *B. anthracis* grows in the presence of oxalates, and so on.

**Magnetism.**—The subjection of suspensions of bacteria to magnetic forces has never been found to exert any appreciable influence on the cells tested.

**Electricity.**—The passage of an electrical current through a bacterial suspension probably has little effect by itself. If a current of great intensity be passed through a culture for a long time, however, electrolysis of some of the constituents of the medium will result, their nature and concentration depending on the voltage, and the composition of the medium and of the electrodes. Some of these products of electrolysis have deleterious effects. Heat, also, will be generated and, if sufficient, may kill the bacteria.

**Electrophoresis.**—The student familiar with colloids knows that any very minute particles, including bacteria, when suspended in

aqueous solutions, acquire an electrical charge on their surfaces. Therefore, when an electrical current is passed through any suspension of charged particles, those with a negative charge will travel toward the positive electrode (anode), while those with a positive charge travel toward the negative electrode (cathode).

The electrophoretic migration of the particles may be observed directly with a microscope focussed on a thin, hollow cell with electrodes at each end (Fig. 48) or by testing for their presence in the legs of a U-tube with electrodes at each leg, the particles being introduced at the middle of the U.<sup>7</sup>

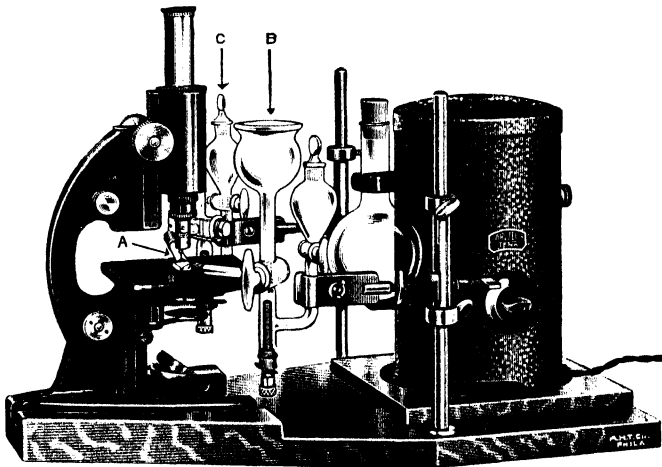


Fig. 48.—One form of electrophoresis apparatus. The microscope is focused on a hollow glass slide containing the suspension of bacteria (A). The electrodes are seen below the stage of the microscope, on either side. The thistle tubes (B and C) and other parts of the apparatus are used for flushing out the cell and changing the fluids around the electrodes. The large canister and flask are concerned with illumination.

This phenomenon has been used in the study of the electrical charge of bacteria and ultramicroscopic viruses and is called *electrophoresis*. For example, it has been shown by Buggs and Green,<sup>8</sup> Dozois and Rauss<sup>9</sup> and others that, by this method, virulent diphtheria bacilli may be differentiated from closely related organisms having little or no virulence, the virulent organisms migrating less rapidly in the electrical field than the avirulent or harmless ones. Verwey<sup>10</sup> has shown that pathogenic staphylococci migrate differently from micrococci of little pathogenicity. Watrous has shown that electrophoretic mobility of typhoid bacilli varies with age and

kind of culture medium.<sup>11</sup> Electrophoresis is an especially useful method in the study of the viruses but requires great skill and a very considerable knowledge of the physics of minute particles on the part of anyone making use of it.

**Desiccation.**—Many vegetative bacteria can withstand complete desiccation for long periods, especially in the dark and cold, if the desiccating process be rapid. However, no growth occurs under these conditions. Some bacteria are very sensitive to cold and slow drying (see evaporation).

*Desiccation and Vacuum.*—Not only do many kinds of bacteria in a vegetative state withstand freezing and desiccation, but they may be subjected to the highest possible vacuums without harm. These facts are of the greatest interest from both a philosophical and practical standpoint.

In a state of complete desiccation and in a high vacuum, there must be an unimaginably small vital activity. The metabolic processes must stop completely since these depend on diffusion, ionization and the colloidal state, all of which are dependent, in turn, on hydration. How, then, can bacteria exist in a vacuum? How can living things survive in the almost entire absence of water and free oxygen or, as it seems, in the entire absence of vital activity? There are no known answers to these questions. The condition of bacteria desiccated in a vacuum must be as near an approach to suspended animation as can be imagined.

If time for human beings be measured by successive events such as heart beats, thought train, action of enzymes, wars, etc., then when these activities cease to be *observed* or *effective*, time ceases for the individual involved and begins again only when the individual consciousness again begins to pace exterior events or when vital activity produces the effects of age. A person deeply preoccupied by study, pain, play or worry, or who has been wholly unconscious, or in an abnormal mental state (delirium), in which consciousness of the course of events is temporarily lost, has a different idea of time from that of normal persons, or at least a very distorted one. An analogous situation exists for bacteria in a preservation vial; although they have no consciousness of passage of time, when the air and moisture are withdrawn from their environment, time may be imagined by us as ceasing to exist for them since their stream of vital activity and the process of aging apparently cease. Time (as measured by us for them) begins again only when air and fluid are added. In the vacuum jar their chronological age may become great, as measured by human standards, but they appear to remain physiologically young and unchanged even for many years during their sojourn in vacuity.

**Preservation of Bacteria.**—From a practical standpoint, the survival of bacteria when desiccated in a vacuum is of great importance. In the laboratory, in the past, it was necessary, in order to

preserve pure cultures of non-spore-forming bacteria, to transfer the organisms frequently to fresh culture medium, incubating and maintaining them in a vegetative state in the refrigerator. The time, labor and cost of such methods can be eliminated where it is known that the species will withstand desiccation *in vacuo*.

Suspensions of pure cultures of such species may be made in a few drops of blood or serum and transferred to a tiny vial containing small discs of filter paper or sterile sand. Such vials are put into a vessel with a small quantity of some desiccating agent (calcium chloride or phosphorous pentoxide) and the highest possible vacuum applied. The vessel is sealed with the vacuum at its maximum. Once sealed in such vessels, a large number of cultures may be stored in a very small space and many bacteria will survive for years. Species of pathogenic streptococci, for example, have survived unharmed for twenty or more years, while diphtheria bacilli have been known to survive for fifteen years and can probably survive much longer under these conditions.

Certain bacteria (meningococci), hitherto supposed to be *very sensitive* to cold and drying, may be preserved desiccated in a vacuum for a year or more after first rapidly freezing them. Pure cultures of tubercle bacilli have been preserved in the same way. It seems likely that prompt desiccation is more important than a high vacuum, although the vacuum is desirable at first in order to insure rapid desiccation. However, while many species of bacteria resist vacuum, freezing, drying and the like, it must be pointed out that this may not be true of all.

*Lyophile Process of Preservation.*—A method of preserving very unstable substances such as living bacteria, filtrable viruses (see Chap. 42), enzymes extracted from the inside of living cells, bacterial toxins, etc., all of which are inactivated or destroyed on prolonged storage in a moist state or exposure to the air, depends on extreme cold and rapid evaporation. The materials to be preserved are suspended or dissolved in an aqueous fluid and distributed in small glass ampoules connected to a high-vacuum pump. The contents of the ampoule are frozen almost instantaneously by immersion in a bath of dry ice in methyl-cellosolve ( $-78^{\circ}$  C.) and then dried from the frozen state by high-vacuum distillation. The necks of the ampoules are sealed with a needle of flame before destroying the vacuum. The product is a highly *lyophilic* (water-loving) powder. A process employing a chemical drying agent, the Chrysochem process, was described by Flosdorf and Mudd,<sup>12</sup> a simplified apparatus by Taylor.<sup>13</sup>

**Preservation of Food.**—Since the activities of bacteria seem to be entirely suspended by complete desiccation, the drying of food and other commodities should prevent decay and spoilage which is due to the active growth of bacteria. The preservative action of drying has indeed been known and used for centuries in the preparation of foods. Dried meats and fish are staples of primitive peoples. Dried apples and plums (prunes), dried grapes (raisins) and other fruits and vegetables “keep” perfectly when dried. The dried milk (“milk powder”) industry is an important one, while hay is a classical example of preservation by drying.

In many drying processes, sunlight is depended upon to aid in effecting the “curing” or preserving action. It will become clear that sunlight has properties which materially aid in food preservation by drying.

**Sunlight.**—It has already been stated that most bacteria do not contain chlorophyll. With the exception of some species of more highly evolved alga-like bacteria found in decaying organic matter where much hydrogen sulfide is being given off (Thiobacteriales), their metabolic processes are independent of sunlight and proceed best in the dark. In early times, it is believed, dense clouds kept most of the sunlight away from the earth’s surface so that whatever chemical processes were made use of by the first forms of life proceeded in its absence. Thus, no chlorophyll or other photosynthetic pigment such as enables green plants to assimilate carbon dioxide under the influence of the sun’s rays existed or could have functioned at that time. Indeed, most bacteria are injured by direct sunlight and species like the bacillus of tuberculosis and a number of others, both parasites and saprophytes, are killed by exposure to the sun for a few minutes or hours. The disinfectant (“purifying”) action of sunlight has been known for centuries but was not until recently understood. It depends almost entirely on the ultraviolet components of sunlight.

**Ultraviolet Light.**—Ultraviolet light has a considerable bactericidal power and it is to this, in great part, that sunlight owes its disinfecting and preserving action. Oxidations are said to be effected by the ultraviolet rays but the action may be due to its effects on respiratory enzymes. Windows and vessels of glass almost completely screen out the ultraviolet rays.

Ultraviolet light, like visible light, consists of a spectrum of “colors.” These consist of different wave lengths of from 4000 Angstrom units down to about 200  $\text{\AA}$  (see Fig. 49). Those from about 2700  $\text{\AA}$  downwards are most effective as bactericidal agents. Radi-

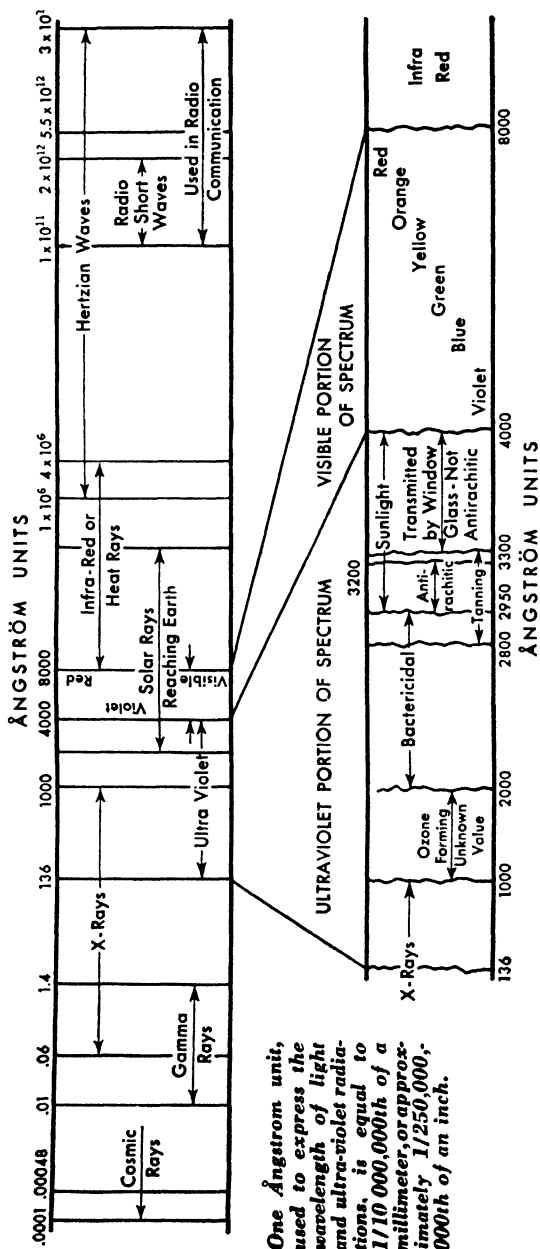


Fig. 49.—Spectrum charts. (From *The Westinghouse Sterilamp and the Rentchler-James Process of Sterilization*, courtesy of the Westinghouse Electric & Manufacturing Company, Inc.)

ating tubes giving off light of wave length about  $2700 \text{ \AA}$  have been put to use in preventing spoilage of meat, tobacco, bakers' goods and the like, due to molds and bacteria. Other uses of the radiations are the disinfection of "conditioned" air, the air in schools, hospitals and operating rooms, and in the control of insects (see section on disinfection). Use is made of ultraviolet light in the treatment of water in swimming pools. It is often applied ineffectively, however, when the water is subjected to its influence for too short a time. This utilization of ultraviolet light promises to be a fruitful field from the standpoint of manufacturers of perishable goods, manufacturers of radiation apparatus and the research microbiologist.<sup>14, 15, 16, 17</sup>

**X-rays, etc.**—Clark, Spencer,<sup>18</sup> and others have shown that radiations of other kinds, like x-rays, and beta rays of radium, have a strongly bactericidal action. Spencer has obtained results indicating that non-lethal exposures to radium induce profound changes in bacteria. Some of these changes involve morphology and other characters to a marked degree. It may be that permanent, genetically stable variants occur under the influence of such radiations. It is possible that this phenomenon, or something akin to it, may be the principal cause of the origin of species, natural selection and evolution being the mechanisms by which these new species are either eliminated as "impractical," or molded into the natural scheme of things.

Darwin pointed out that fitness of new, genetically stable variants to meet the environmental conditions imposed by Nature (natural selection) was the *sine qua non* of survival of the new type or species, and his conception of the origin of species was based on this idea. However, the *origin of the mutants*, the real, original, origin of species, escaped him and may have as its basis genetic changes induced by various influences such as radiations.

**Sound.**—Bacteria, because of their small mass and cell wall, are not readily disrupted by vibration. Sound vibrations within ordinary ranges from very low up to very high rapidities apparently have little effect as a rule. The "high" limit of audible tone has a vibration rate of around 30,000 per second, the lowest audible tone has a vibration rate of around 20 per second. However, a sonic vibrator consisting of a nickel tube, motivated by means of an electromagnetic field, has been devised which emits sound waves with a frequency of 9,300 vibrations per second. This particular tone is lethal for some species of bacteria, as well as one type of bacteriophage.<sup>19</sup> *Supersonic vibrations* are so rapid that even bodies having



the small inertia of bacterial cells are shaken asunder when subjected to their influence.<sup>20</sup>

These methods of shattering bacteria are of value in experiments where it is desired to kill and disrupt the organisms without producing such profound chemical and physical changes as result from heating, chemical destruction, exposure to sunlight, or age. Delicate poisons, proteins, carbohydrates or enzymes inside the cells have been liberated in an unaltered state by supersonic vibrations and may thus be studied in an unchanged condition.<sup>21</sup> Particles as small as viruses may be very resistant to vibrations.<sup>22</sup>

**Rapid Freezing and Thawing.**—Bacteria are also broken up by a rapid alternation of freezing and thawing. The freezing is usually

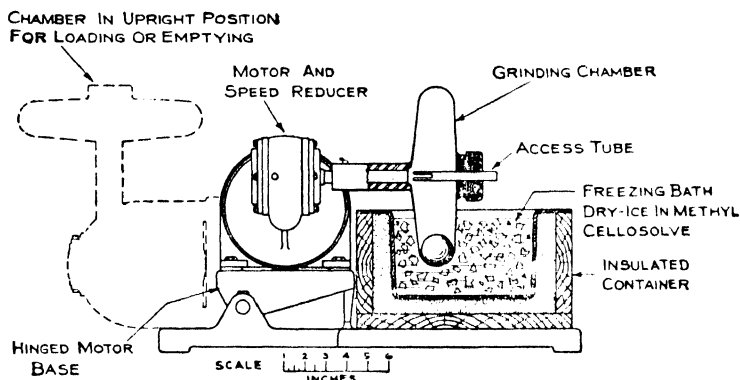


Fig. 50.—A freezing ball-mill. (Mudd, Shaw, Czarnetsky and Flosdorf, U.S.P.H.S. Weekly Reports, Vol. 52.)

accomplished by immersion of the vessel containing the bacterial suspension in alcohol containing blocks of solid carbon dioxide ("dry ice"). The thawing should be at a temperature of not over 45° C. or coagulation may result.

**Pressures.**—Bacteria can withstand extremely high pressures (90,000 pounds or more per square inch) of gas or fluid, but are easily crushed by the impingement upon them of solid particles like steel or glass balls. Shaking in a round vessel with steel balls (ball-mill) is a method used for the mechanical destruction of these organisms. They shatter especially readily if made brittle by freezing and this method is used for certain chemical investigations of bacteria (Fig. 50). Shaking for several hours of a bacterial suspension mixed with extremely fine beads of glass ("glow beads") has been

shown by Curran and others to accomplish a complete disruption of the cells.<sup>23</sup>

**Photodynamic Action.**—Bacteria will often grow in contact with low concentrations of bacteriostatic dyes such as eosin, fuchsin, etc., provided no light reaches them. The combined effect of light and dye for only a few minutes usually kills the organisms. As shown by Tung and Zia,<sup>24</sup> methylene blue, in the light of an ordinary electric lamp, rapidly kills certain gram-positive bacteria which withstand much higher concentrations of the dye in the dark. Gram-negative bacteria are not so affected.

**Osmotic Pressure.**—Bacteria are, as a rule, not very sensitive to variations in salt concentrations below about 2 per cent. Concentrations much above this may adversely affect some of the more sensitive strains. Bacteria seem quite variable in this respect. ZoBell and others have shown that marine bacteria, adapted to the salinity of ocean water (about 3.5 percent) are quite sensitive to lower salinities and will not grow if the salinity is less than about 2 percent. On the other hand, if media intended for colony formation by bacteria adapted to life in fresh water lakes be given a salinity as high as 1 percent, no colonies will be formed by certain aquatic species.

As shown by ZoBell there are bacteria which have become adapted to the high salinity (27.6 percent) of the Great Salt Lake of Utah. These organisms will not grow in lake water diluted to a salinity of less than 13 percent. Such organisms are spoken of as *halophilic*. On the other hand, the growth of soil, sewage or mouth bacteria is inhibited by the lake water and ordinary marine bacteria are killed by a few minutes' exposure to it. There are halophilic bacteria which grow in commercial pickling brines. Some are a cause of spoilage of various commodities preserved with salt, such as fish, meat, and hides. Concentrations of salt around 25 and 30 percent are used in these brines.<sup>25, 26</sup>

**Drug Fastness.**—A good example of bacterial adaptation is seen in the acquisition, by many species, of ability to grow well in solutions having salinities quite different from that to which they are accustomed. The change in osmotic conditions must be gradual. Similarly, many organisms will adapt themselves to growth in the presence of poisonous substances like phenol if minute amounts, and later increasingly larger amounts, of the poison be introduced into successive subcultures. Bacteria adapted to growth in the presence of such toxic drugs are said to be "drug fast." Adaptability of certain pathogenic species to sulfonamid drugs often interferes

in the therapeutic use of these compounds and a rotation of drugs (successive use of various kinds) is resorted to.

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## CHAPTER 6

### STERILIZATION AND DISINFECTION

#### MECHANISMS INVOLVED

THE BACTERIAL cell, like all living cells, depends for its life and normal functioning in great part on the maintenance of its protoplasm or cell substance and its enzymes in a properly reactive condition. This, in turn, depends on a continuance of its chemical composition and the maintenance of colloids in a state of equilibrium. When any of these factors is disturbed, growth is interfered with and life may cease if the disturbance is sufficiently severe or prolonged. Advantage has been taken of these points of vulnerability of bacteria to perfect methods of killing them or inhibiting their growth. One type of method is directed primarily toward alteration of the *chemical composition* of the protoplasm, or of some particular vital constituent thereof, for example a respiratory enzyme. Another type consists in destroying the *colloidal state* of the protoplasm. Some methods in common use accomplish both the chemical and physical type of change; the action of others is not wholly understood. We shall first discuss methods of producing physical changes in the protoplasm. These affect mainly its colloidal state.

**Colloids.**—A substance is in a *colloidal state* (sometimes called the fourth state of matter) when it occurs in such finely divided particles (the disperse or discontinuous phase) that they remain suspended in gas, water or other fluid (the continuous phase) in-

definitely. Such suspensions in fluids may be clear or "smoky" or milky in appearance but have, in general, a superficial resemblance to true solutions. An example of a colloidal suspension in a gas is smoke. Smoke is composed of minute particles suspended in air. Milk is a colloidal suspension of casein and butter-fat in whey. Many substances, including various metals, can be made to assume the colloidal state. Colloidal particles vary in size but are probably made up of small groups of molecules or atoms. Their small size is important because when a given weight of a substance is subdivided into very tiny particles its surface is greatly increased and, as we shall see, many biological phenomena depend on chemical and physical reactions occurring on surfaces.

**Surfaces of Colloids.**—A cube measuring 1 cm. along each edge presents a surface of 6 sq. cm. Cut in half, 8 sq. cm. are presented. In 10 slices, 24 sq. cm. are obtained, while if cut into particles almost as small as molecules, the surface is relatively enormous. Thus, a very small volume of matter may, by being extremely finely divided, acquire a very large surface. This is the state of affairs with colloids. This surface development is very important, since forces active at surfaces greatly affect colloids.

**Protoplasm a Colloid.**—Protoplasm (roughly all the living cell substance enclosed in the cell membrane) is principally proteinaceous matter in a colloidal state, the minute proteinaceous particles being suspended in an aqueous matrix containing, in solution, various salts, organic substances, nutritive compounds which have diffused inward from the surrounding medium through the cell membrane, and waste substances, toxins and the like which will diffuse outward and be liberated in the medium. Between the colloidal particles and the suspending fluid inside the cell there is a constant interplay and exchange of various atoms, molecules, electrical forces, physical stresses, chemical attractions, and so on, all in a delicately balanced state of equilibrium with respect to each other, the net resultant of all being life, reproduction, motility, fermentation, putrefaction, luminescence, toxin production, and all the phenomena familiar to the bacteriologist. Obviously, any abnormal alteration, however slight, in the physical or chemical constitution of the contents of the cell will profoundly affect the functioning of the cell and may result in its death. It is necessary for the bacteriologist to be informed as to the agents which bring about such changes, both that he may avoid them in attempting to cultivate bacteria, as shown in the next chapter, and that he may make use of them intelligently in disinfection and sterilization.

**Protein Coagulants.**—It is well known to every informed person that bichloride of mercury is a good, general disinfectant. The reason for this is not so commonly understood. It is, however, quite simple if we remember the dependence of life upon the proper colloidal equilibrium of the cell contents. If these be thrown out of their watery suspension, or *coagulated* into a firm mass, as occurs when eggs are “hard-boiled,” milk soured or “junket” made, life ceases. Bichloride of mercury is one of a number of substances which coagulate protein rapidly, and that is one reason why it is a good disinfectant. It also has more complex actions of an obscure, and apparently reversible, physicochemical nature. Phosphotungstic acid, carbolic acid (phenol), formaldehyde, alcohol, copper sulfate and salts of a number of other heavy metals also coagulate protein more or less readily and all, therefore, have a *bactericidal* action. Heat also coagulates protein. The general rule may be stated, then, that *any substance or condition which results in the coagulation of protein is fatal to bacteria.*

Coagulation by heavy metal salts or acids is largely an electrical phenomenon. The colloidal particles of protein are negatively charged. Being similarly charged they repel each other, do not clump, and remain in suspension. Metal and H ions are positively charged. These neutralize the negative charges of the protein colloids, permitting clumping, and coagulation occurs. That the process is not *wholly* electrical is shown by the very different actions of different kinds of metals. Coagulation by organic compounds such as phenol is of a different sort from that produced by metals, and is not completely understood. It is probably caused by a combination of chemical and physical effects (see discussions of *pH*, page 83 and of action of salts, page 88).

**The Rôle of Hydration in Protein Coagulation.**—The rôle of *hydration* or water content in protein coagulation is an extremely important one and is clearly shown by experiments on the effect of heat on egg albumen under various degrees of hydration. The results of some of these experiments are shown in the following data collected by Frost and McCampbell.<sup>1</sup>

Egg albumen + 50 percent water coagulates at.....	56° C.
Egg albumen + 25 percent water coagulates at.....	74°– 80° C.
Egg albumen + 18 percent water coagulates at.....	80°– 90° C.
(boiling water = 100° C.)	
Egg albumen + 6 percent water coagulates at.....	145° C.
Egg albumen + 0 water coagulates at.....	160°–170° C.
(oven temperature)	

Obviously coagulation proceeds best where the protein is in its usual, actively living, well-hydrated or wet condition. The less the degree of hydration, the more resistant is the protein to coagulation. The same principle holds true in coagulation by chemicals. The resistance of bacterial spores to heat and chemical disinfectants may be explained as being due to the dehydrated condition of the protein or protoplasm within them. Examples of the resistance of dehydrated protein to the action of coagulative agents are seen in the difficulty encountered in making dried-egg powder coagulate. Heated in a test tube over a naked Bunsen flame, it will turn brown or char, but not until it has soaked up a considerable quantity of water will it coagulate. Of course, mixing with dry chemical disinfectants such as powdered bichloride accomplishes nothing at all till moisture is added. The same is true of dried-milk powder. It would be difficult indeed to make "junket" or cottage cheese without plenty of water.

For these reasons *aqueous* solutions of disinfectants which coagulate are generally more useful than solutions of the same substances in solvents other than water. Alcohol, in 70 percent solution in water, for example, is more effective than pure, absolute alcohol containing no water, since it hydrates the protein to be coagulated.

### PURPOSES AND DEFINITIONS

There are three main reasons for killing or removing bacteria. They are: (1) to prevent infection of men, animals and plants; (2) to prevent spoilage of food and other commodities; and (3) in research, in order that studies of the growth of one kind of organism in a particular medium or infected animal may not be confused by the presence and growth of others at the same time.

There are several substances and procedures involved in accomplishing these results. These may be defined as follows:

**Sterilization.**—Sterilization means the freeing of any object or substance from all life of any kind. This is accomplished usually by heat but sometimes, in special cases, by the use of chemicals, x-rays, radium, etc.

**Bactericides.**—Any substance or agent killing bacteria is a bactericide or bactericidal agent. The suffix, *-cide*, indicates "killer" and is used with germ, virus, etc.

**Disinfection.**—Disinfection, as the word implies, means the killing or removal only of organisms capable of causing *infection* and does not necessarily require that *all* organisms be killed. However, many processes of disinfection actually accomplish sterilization.

Disinfection is usually accomplished by chemicals like carbolic acid, formaldehyde, iodine or bichloride of mercury. In the case of milk, disinfection, *but not sterilization*, is brought about by *pasteurization*, a heating process to be described.

A *disinfectant* is an agent accomplishing disinfection. The term is often used synonymously with *antiseptic*.

**Asepsis.**—In a strict sense, asepsis is the practice of keeping *infectious* bacteria away, but the term is usually applied to any technic designed to keep *all* bacteria out of the field of work or observation. The work of a bacteriologist and of a surgeon involves aseptic technic. The surgeon and his assistants have sterile instruments, handle them with sterilized gloves, cover the patient with sterile sheets except for such area as is necessarily uncovered, and wear sterile caps, gowns and masks to prevent infected dust, droplets of saliva, perspiration or sputum from entering the sterile field and possibly infecting the patient. The patient's skin cannot be absolutely *sterilized* without injury, but the site of the operation is disinfected as thoroughly as possible by applications of iodine or some other suitable *disinfectant*.

In bacteriology the worker uses *sterilized* culture media and *sterilized* glassware kept sterile, until the moment of use, by coverings of paper and cotton plugs and by *aseptic technic*.

#### METHODS: I. STERILIZATION BY HEAT

Heat may be applied for sterilization in three ways: (a) by steam or hot water (moist heat); (b) by prolonged baking in the oven (dry heat); and (c) by complete incineration. The last needs no comment beyond pointing out that common sense will direct what may be burned up and what should not.

**Boiling in Water.**—The use of hot water for sterilization and disinfection is very simple. It is only necessary to remember that many bacteria and molds form heat-resistant spores, some of which may remain alive even after an hour of boiling. For ordinary household purposes of *disinfection*, but not *sterilization*, 5 minutes' boiling of dishes, clothes, etc., is usually sufficient, provided the hot water actually comes into contact with the bacteria, and not merely into contact with the outside of lumps or packets or other objects containing them. In *all* disinfection and sterilization, *contact* between the bacteria and the agent used is absolutely essential.

**Free-flowing ("Live") Steam.**—"Live" or free steam is usually applied in an apparatus like that shown in Figure 51. Boiling water and free steam never reach a temperature above 100° C. (212° F.).



In mountainous regions it is much lower than  $100^{\circ}\text{C.}$ , as at Tahoe, California, elevation about 10,000 feet, where boiling occurs at about  $94^{\circ}\text{C.}$  Free steam is generally used to accomplish *fractional sterilization* or *tyndallization*. It is usually employed in preserving foods or culture media or other "putrescible fluids" which are not amenable to *autoclaving* or to baking in an oven, in which much higher temperatures are used. For example, prolonged heating of carbohydrate solutions in preparation for fermentation tests causes

hydrolysis of many of them. Autoclaving is preferable in this case. Some substances are precipitated or otherwise injured by autoclaving.

*Tyndallization.*—Tyndall, a famous British biologist who was much interested in bacteriology, noticed that some bacteria have two phases. At one stage they are relatively sensitive to heat and are destroyed by boiling ( $100^{\circ}\text{C.}$ ) in 1 minute or less; this is the actively growing or *vegetative state*. In the other stage, he observed, they are dormant and very thermoresistant. This latter is the stage or form known as the *spore stage*. Tyndall observed also that a period of about 12 to 18 hours' incubation at ordinary room temperatures is necessary to enable the dormant and heat-resistant spores to germinate and pass into the active or vegetative state in which they are sensitive to heat. He devised a process of sterilization based on these observations, which consists in steaming for a few minutes on three

or four successive occasions separated by 12- to 18-hour intervals of incubation at favorable temperature. The intervals permit the dormant spores to become vegetative. This process renders an infusion sterile whereas one single, continuous boiling for 1 hour may not, since a few of the spores remain in their dormant and resistant state during this time. This intermittent process is frequently used today in the laboratory and is put to practical use in the home canning of foods. A great disadvantage of the method is the time required to achieve complete sterilization. An advan-

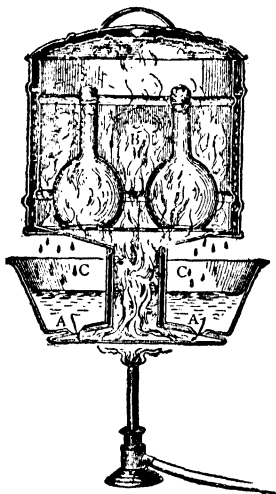


Fig. 51.—Arnold sterilizer used for tyndallization. Water from *C* passes through holes (*A*), comes into contact with the heated bottom of the vessel and rises as steam into the chamber *B*, where it condenses and drips back into *C*.

tage is that it requires no special apparatus. In some fluids, as water, spores may not germinate promptly. Also, if the material is freely exposed to air, anaerobe spores may not germinate.

**Compressed Steam. Autoclaving.**—Anyone familiar with the operation of a pressure cooker (Figs. 52 and 53) is familiar with an *autoclave* because the cooker is a simple form of autoclave. In the autoclave (Figs. 54 and 55), be it a small and simple home pressure cooker or an apparatus large enough to fill a room and fitted with

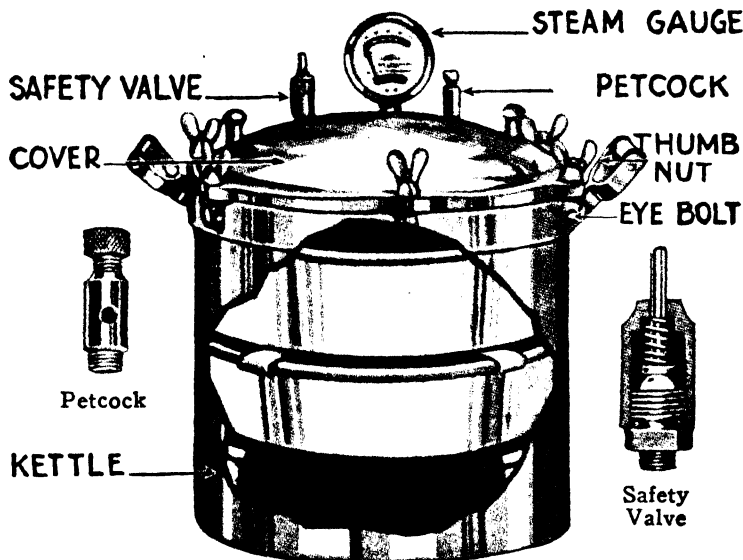


Fig. 52.—A modern type of pressure cooker. About 1 quart of water is placed in the bottom of the kettle. When it boils, steam forces air out through the petcock which is left open till pure steam emerges. When it is closed, pressure is registered on the steam gauge and, if the flame is not lowered, will escape through the safety valve. The thumb nuts and eyebolts are used to tighten and hold the cover. Details of the petcock and safety valve are shown in the insert.

various gauges, pipes, valves, clocks and wheels, the object is to heat the articles to be sterilized by means of steam under considerable pressure.

Steam under pressure is hotter than boiling water or free-flowing steam such as is used in tyndallization or the Arnold sterilizer. The higher the steam pressure, the higher the temperature. It must be remembered, however, that it is the compressed *steam* (moisture, *hydration*) that sterilizes and not compressed air (dry and usually

not as hot as steam). Steam *hydrates* and thus promotes coagulation. Air does not. Steam, being water vapor, also produces hydrolysis at autoclave temperatures. Air cannot do this at any temperature. In autoclaving, therefore, as in using a pressure cooker, a valve is left open for the escape of all air *before the steam pressure is allowed to rise* (Fig. 54 A).



Fig. 53.—Front Line Surgery—The same sort of pressure cooker used by housewives in the United States for putting up the vegetables and fruits from their victory gardens serves at the front lines in China to sterilize instruments. This photograph is furnished by United China Relief, which organization is providing funds for such emergency medical work. (Science Service.)

The actual amount of water present as steam in an autoclave is small and articles soon dry off after removal, especially if removed from the autoclave while hot.

The *thermometer* on the autoclave is the important guide to the process of autoclaving and not the *pressure gauge*, although the latter, as well as a steam-escape or safety valve, is desirable for *safety*.

The common practice in autoclaving fluids or freely exposed surfaces such as those of dishes and instruments is to apply  $115^{\circ}$  to

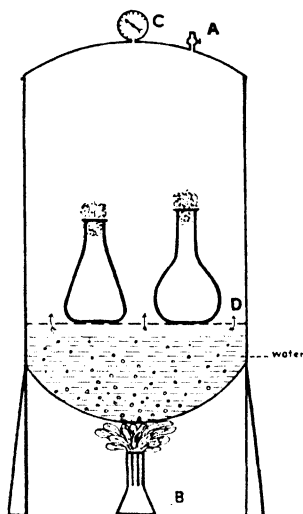


Fig. 54.—Diagram showing structure of a pressure cooker or simple form of autoclave. At A is shown the valve used to allow the escape of air. C is a combined pressure gauge and thermometer. D is a shelf, perforated to allow the steam to rise from below. (Park and Williams, "Pathogenic Microorganisms," Lea and Febiger, publishers.)

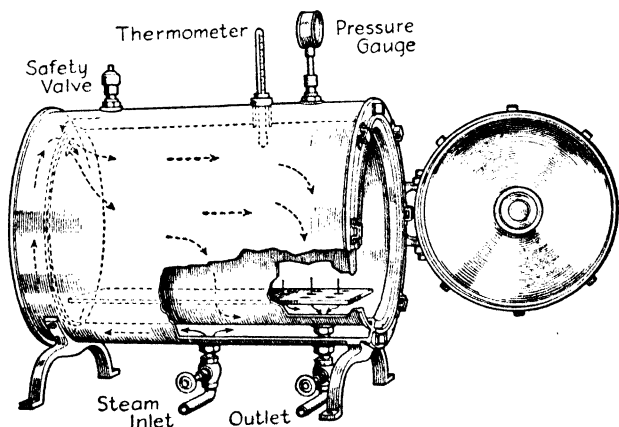


Fig. 55.—Autoclave of modern double-jacketed type, showing pathways of steam and essential parts. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

125° C. (10 to 20 pounds' pressure) for 20 minutes. The pressure must be allowed to subside *slowly* after the heating is over or *super-*

*heated* fluids in the vessels will boil over. Any large, solid masses must be heated a longer time, to allow for heat penetration. Packages should be spaced so as to allow free circulation of steam. Substances like mineral oil or vaseline, sand, etc., or any dry objects in tight jars, or which are *impervious to moisture*, cannot be satisfactorily sterilized in the autoclave. The *temperature* may rise high enough but, in the absence of moisture, is ineffective. Such materials are more effectively sterilized in an oven.

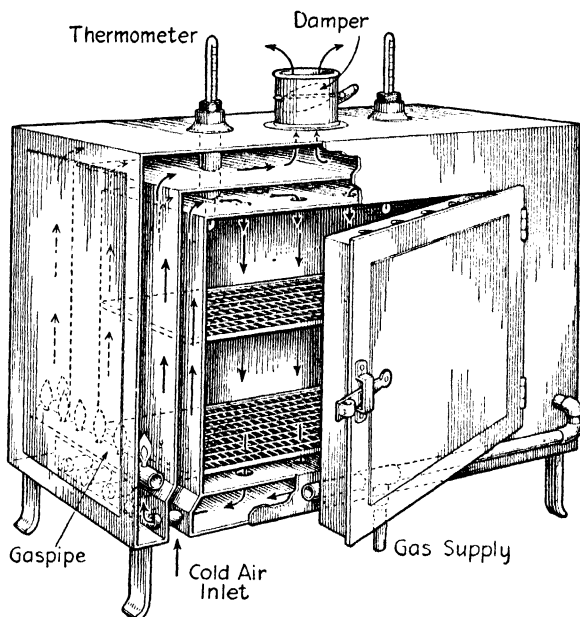


Fig. 56.—Hot-air sterilizer. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

**Dry Heat.**—Dry heat is used in oven sterilization (Fig. 56). It is necessary to bear in mind that moist heat, however applied, kills bacteria by coagulating their protein just as hot water coagulates an egg, and that only thoroughly hydrated protein coagulates readily. Coagulation does not occur when moisture is not present.

Articles in ovens are very dry and therefore, in order to be freed of live spores, must reach a very high temperature ( $165^{\circ}$  to  $170^{\circ}$  C.;  $329^{\circ}$  to  $338^{\circ}$  F.). It is customary to apply  $165^{\circ}$  C. for a period of 2 to 3 hours. This accomplishes not only coagulation, but, what is more effective, slight charring.

A home oven can easily be used for sterilization. A moderate temperature (330° F.) is satisfactory, and the process should be allowed to proceed for 3 hours. Paper wrappings should be browned but not brittle; muslin or string should be yellow or light brown, due to the heat.

Only dry articles not injured by baking (glassware, bandages, instruments, mineral oils, vaseline, and the like) may be thus sterilized. Solutions containing water, alcohol or other volatile substances will, of course, boil away and be ruined.

**Thorough Heating Necessary.**—In any process of disinfection or sterilization by heat it is absolutely essential that the object be heated through and through and that the *center* of the object be held at a killing temperature long enough to destroy the bacteria.

Thus, in canning, a quart jar of spinach may be held in free steam (100° C.) for 5 or 10 minutes and when grasped with the hand will feel very hot. It has been shown, however, that large masses of nonfluid materials like quart jars of canned vegetables and roast meat, in which the contents cannot *circulate*, require a long time (1½ to 2 hours) even in the autoclave to be heated so thoroughly that the center reaches a bactericidal temperature. Penetration of the heat is facilitated if the cans or jars or pieces of roasting meat, etc., be small and not packed too closely together, facilitating free circulation of steam or hot air around and between them.

## METHODS: II. CHEMICAL DISINFECTION

The physics and chemistry of chemical disinfection are extremely complicated. Much depends on the disinfectant used. We shall discuss here only a few types of disinfectant which illustrate most of the important principles involved. These disinfectants are of value for external application, household use and the like. The antibacterial action of sulfonamid drugs, penicillin, etc. will be discussed farther on.

Carbolic acid and the cresols represent a type of disinfectant which acts by coagulation and also by certain little-understood chemical combinations. These are surface-active, *cationic*, organic disinfectants.

*Bichloride of mercury* represents a chemically different class of coagulative disinfectants, being a simple, inorganic, non-cationic compound having little surface action; *i.e.*, not a surface tension reducent.

Disinfectants acting largely by chemical action are typified by iodine, chlorine, "lye," hydrogen peroxide and potassium perman-

ganate. The last two give off "nascent" oxygen which, presumably, oxidizes the bacteria or some sensitive constituent of their protoplasm.

*Chlorine and iodine* form compounds, combining with the constituents of the protoplasm so that the latter ceases to exist as such. They also oxidize, as they liberate "nascent" oxygen from water:



The acid also has a bactericidal action.

"*Lye*," which consists chiefly of potassium hydroxide, destroys many organic substances including protoplasm by virtue of its powerful caustic or corrosive action.

*Strong acids* coagulate, and act by corrosion as well. The mechanisms of these actions have been discussed in the section on the effects of environment on bacteria (see page 83).

*Certain dyes* have the power of combining with bacterial cells and killing them. Crystal violet is often used as an application on skin infections. Gram-positive bacteria are especially susceptible to it. Other dyes, such as malachite green, have special affinities for particular species of bacteria. Practical uses of this will be mentioned later (see page 126). Dyes are cationic disinfectants and probably act by combining, after adsorption, with certain groups, possibly carboxyl groups, of proteins. Sometimes these combinations are reversible and the bacteria are capable of being "revived"; i.e., the combination is not in itself lethal unless continued till the organisms die from other causes. This is bacteriostasis and is discussed below.

**Disinfection and Contact.**—Like hot air and steam, any chemical disinfectant, in order to be effective, must come into contact with the bacteria to be killed. This seems self-evident yet, in practice, it is not always easy to accomplish this contact and it is often overlooked or neglected. There are several factors of importance with respect to bringing about this necessary contact. One of these is surface tension.<sup>2, 3, 4</sup>

*Surface Tension.*—It will be remembered that there exists an attraction or cohesive force between molecules. The attraction exists in all matter and is evidenced, in fluids, by the action of their surfaces. It may be illustrated as follows:

If we imagine a single molecule in a beaker of pure water (Fig. 57, a), a few centimeters below the surface, it is obvious that the attraction of the surrounding molecules is equal on all sides. When

this molecule is at the surface (Fig. 57, *b*), the attraction of the large number of liquid molecules in the water below is much greater than that of the few in the vapor above the surface of the liquid. The same is true of the molecules at surfaces in contact with the glass walls of the vessel or any particle suspended in the water; *i.e.*, the molecular pull is from only one direction. It is easy to see that the pull upon all the molecules at the *surfaces* of the liquid is greatest from below, or within the bulk of the fluid, and that the surface of the fluid mass will therefore tend to be drawn in. It is this force which enables us to fill a beaker to a level several millimeters above the actual limits of the glass walls. The intermolecular pull results in *surface tension*. Under its influence the fluid surface acts, in many respects, like an elastic membrane.

Study has shown that protoplasm is colloidal and that colloids have large areas of surface per unit of volume. Bacterial cells themselves are so small as to have many of the surface characteristics of colloids. Surface-active forces, therefore, play a relatively large role in the vital activities of the cell. It may be helpful to consider some of the more important changes which can be wrought in the physical and chemical state of various objects, especially at the surfaces of bacterial cells and, as will be shown later, of various catalytic agents, by surface forces.

**Adsorption.**—Of the many phenomena which result from the action of surface forces, probably the most important and best understood is *adsorption*. Explanations of adsorption and surface tension are based on the *law of entropy*. This law requires that any change which can reduce the amount of free energy in the universe shall tend to take place to a maximum degree. Surface tension is a form of free energy. Suppose a substance (*e.g.*, soap) which reduces surface tension is added to the water in the beaker shown in

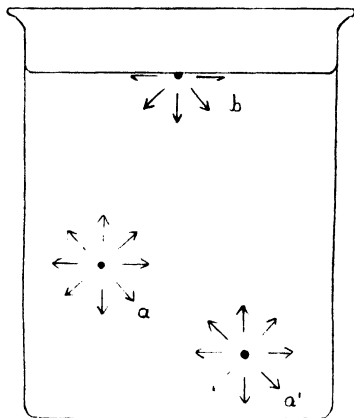


Fig. 57.—Diagram showing how surface tension acts. The molecules *a* and *a'* are attracted equally from all directions and are in a state of equilibrium. Molecules at the surface, like *b*, are under a greater tension from below than above and the entire surface therefore tends to pull inward much as though the surface film were a rubber membrane.



Figure 57. It must, according to the law of entropy, tend to carry the reduction of surface tension as far as possible. This may be accomplished in several ways. For example, the actual extent of the surface may be reduced by contraction or shrinkage in volume. Should this be impossible, as in this case, the surface tension reductent can lower the amount of free energy to the greatest extent only by accumulating at the surfaces of the fluid. This accumulation is known as *adsorption*. Deposition of the surface tension reductent may then occur on the air-fluid surface, at the surface in contact with the glass walls of the containing vessel and on the surface of any particles, such as bacteria, suspended in the fluid.

It has been shown that substances adsorbed upon a surface are often greatly *concentrated* and that, in many cases, their *solubilities increase*. It is probable that some gases are actually liquefied when adsorbed. It is clear that such condensations and increases in solubility must, from the *law of mass action*, greatly facilitate chemical reactions, since the law of mass action states that the greater the concentration of the reacting substances the more rapid the reaction between them. Disinfectants, especially cationic, surface-active substances, and catalytic agents discussed in the chapter on enzymes (see page 334) depend upon this law, which governs molecular contact.<sup>4a</sup>

As will be seen later, the vital chemical activities occurring in and on living cells are also brought about through the effects of adsorption and the phenomenon should be well understood at this point.

As previously mentioned, bacteria are electronegative. Cationic substances, including disinfectants like alkyl-dimethyl-benzyl-ammonium chlorides, therefore tend to combine with them as a result of electrical attraction.

**Contact and Surface Tension.**—Upon adding carbolic acid to a broth culture of bacteria, contact between disinfectant and bacteria is immediate. The bacteria float naked, as it were, and are reached by the disinfectant in effective concentration without delay partly because *carbolic acid lowers surface tension* and is adsorbed upon their surfaces. If the bacteria are enclosed in a large mass of sputum or feces, only by a thorough mixing process does the disinfectant reach all of them, since the forces resulting in adsorption are effective only through distances molecular in magnitude. The same applies to electric forces which in part determine the value of cationic disinfectants.

**Wetness.**—An important result of the power of substances to lower or weaken surface tension lies in the fact that disinfectant solutions having low surface tension spread better, *i.e.*, are actually wetter, than other solutions. Mercury, for example, although a fluid, has a very high surface tension and therefore, as everyone who has held mercury in the palm of the hand knows from experience, possesses the property of wetness only in a very slight degree. Water also has a relatively high surface tension, while alcohol has a much lower surface tension. Alcohol wets, or spreads when in contact with surfaces, much better than does water and thus comes into more intimate contact with objects which it touches.

An illustration of the superior spreading properties of a disinfectant solution with low surface tension or wetness is seen in the action of an alcoholic solution of iodine as compared with that of an aqueous one. If crystalline iodine be added to an aqueous solution of potassium iodide, it will dissolve readily and can be brought to the same strength as the alcoholic solutions sold in every drug store as "tincture of iodine." Put a drop of such an aqueous solution of iodine on the leveled back of the hand or on a table top. It remains in a droplet, does not tend to spread very far, and does not, unless "rubbed in," come into very intimate or extensive contact with the skin or crevices and pores in the wood. A drop of the alcoholic solution, on the contrary, spreads immediately and appears to soak into, and to wet thoroughly, the material being disinfected. Alcohol is thus seen to be much wetter than water from a physical standpoint (as well as in a political sense). If organisms be coated with an oily or waxy film, as is the case naturally with tuberculosis bacilli, even when a disinfectant reaches them it affects them with difficulty unless it possesses the special physical property of lowering surface tension, thus allowing it to *wet* the wax.

**Application of Disinfectants.**—Solutions of disinfectants in dilute alcohol or in similar substances such as acetone, etc., which have low surface tensions, are thus much more likely to be effective than when dissolved in water, although water cannot be eliminated entirely because of the necessity for hydration in disinfectant action. If pure alcohol be used as a solvent, disinfectants which kill by coagulation alone may lose efficiency because of the absence of water or *hydration*. Alcohol, in itself a mild disinfectant of the coagulative type, is, therefore, when used as a handwash, generally prepared in a 60 to 80 percent concentration. Bichloride of mercury is usually applied in aqueous solution so that moisture is available although the addition of some liquid surface tension reducent

like alcohol greatly increases the effectiveness of such solutions. Also, if the materials to be disinfected are dry to start with, long soaking with the aqueous solution is necessary in order to be sure of hydration.

Coagulative disinfectants, like bichloride of mercury, often defeat their own object when used for the disinfection of sputum or wounds where there is much coagulable material (pus or serum). They coagulate the sputum or serum around the bacteria so that the disinfectant fails to reach them. Disinfectants which are not so highly coagulative but which act by chemical effect, at least in part, are preferable. In such situations one would choose chloride of lime, or a low-surface-tension solution of phenol.

**Soap and Disinfection.**—Soap, in itself a mild disinfectant with respect to certain bacteria, has, like alcohol, the power of lowering the surface tension of water and this not only induces the soap to be adsorbed on the surfaces of the bacteria but also permits the water to wet surfaces more thoroughly. Carbolic acid and related compounds, like cresol, have these properties also and are, in and of themselves, powerful germicides besides. A combination of *liquid* soap, carbolic acid and cresols would, therefore, seem to have exceptional possibilities as a disinfectant. Indeed, mixtures containing these substances in *effective proportions* are widely used in hospitals, laboratories, etc. A product of this type is available on the market, or can be made up as *liquor cresolis compositus* from the U. S. Pharmacopeia.

Solutions of certain high-molecular dimethyl-benzyl-ammonium chlorides, which are cationic, surface-active disinfectants have similar wetting properties.<sup>5, 6</sup> In addition they have “long-chain” organic radicles which, in general, increase the effectiveness of such compounds as well as of alcohols, resorcinols, etc.<sup>7, 8</sup> An excess of *solid* soap will interfere with the adsorption of the disinfectants. In general, the addition of a relatively ineffective, very surface-active substance like soap to disinfectant solutions is likely to be disadvantageous because the soap may be adsorbed to the exclusion of the more effective substance in the mixture.<sup>3, 8a</sup>

**Antagonistic Action of Toxic and Nontoxic Substances. Bacteriostatic vs. Bactericidal.**—Although, as shown below, we differentiate between bacteriostatic and bactericidal actions, the line of demarcation is not well defined. For example, *Staphylococcus aureus* will not grow in the presence of 1 percent mercuric chloride, and after several hours' exposure may be killed! This might be called disinfection. But if the drug be removed from the organisms

by washing after as long as two hours' exposure, the cocci will be found viable although no growth would occur before the washing. This would be called bacteriostasis. The difference between bacteriostatic and bactericidal action in this case seems to be a matter of time and personal opinion. If a substance such as hydrogen sulfide is used for detoxication, the organisms may be revived after seventy-two hours' contact with the bichloride. Constitution of environment as well as time obviously must be considered in distinguishing bactericidal from bacteriostatic actions. In this case the detoxifying agent destroys or inactivates the toxic substance by chemical combination and bacteriostatic action of the bichloride is clearly shown. Various antagonistic actions may exhibit the same phenomenon. Antibacterial action would therefore seem a preferable term in place of bacteriostatic and bactericidal.

Another type of antagonistic action has been demonstrated by Valko and DuBois<sup>8a</sup>, who showed that the antibacterial action of certain toxic surface-active cations could be reversed by the use of similar cations of low toxicity. Using *Zephiran*, a mixture of high-molecular alkyl-dimethyl-ammonium chlorides as a toxic, surface-active cation, it was found, for example, that the toxic action could be reversed with sodium dodecyl sulfate (Duponol PC), a soap-like, surface-active cation of relatively low toxicity. *Staphylococci* treated with 1 : 3000 *Zephiran* could be revived by treatment with the dodecyl sulfate after ten minutes but not after thirty minutes, provided sufficient detoxifying agent were used and the two drugs together were not in contact with the organisms too long.

The experiments showed also that adsorbability (contact) is a determining factor in the effectiveness of an antibacterial surface-active cation, after which the specific toxicity of the cations is a contributing factor. In the example given above, which is representative of a general type, the antagonistic action of the *less* toxic ion is probably due to competitive adsorption—the harmless cations displacing many of the toxic cations from the available spaces on the bacterial surfaces. This would assume an approximately equal adsorbability of the two types of ion, or a greater adsorbability of the harmless ion if the antagonistic effect is well marked.

In all such phenomena *pH* plays an important rôle. Increased alkalinity favors surface-active cations and acidity favors anions in regard to both gram-negative and gram-positive organisms.

These phenomena, based on competitive adsorption, are reminiscent of the antagonistic relationship between sulfonamid drugs and *para*-aminobenzoic acid discussed elsewhere in this book (see

page 128) although the exact mechanism is not the same in both. Herrell and Heilman state that the antibacterial action of gramicidin (see page 141) is inhibited by a cationic detergent called *Phemerol*. Penicillin (see page 132) appears not to be inhibited by either gramicidin, *Phemerol* or *Zephiran*.

**Time, Temperature and Concentration.**—Disinfectant solutions are not magical. Their values differ in different situations, depending on the kind of bacteria to be destroyed and their location (open wounds, skin, floors, dishes, clothing). No matter what disinfectant is selected for use under any given circumstances, three important factors play a part in the results. These are:

A. *Concentration*

In general, the more concentrated a disinfectant, the more rapid and certain its action.

B. *Time*

No disinfectant, as ordinarily used, acts instantly. Sufficient time for contact, and for whatever chemical or physical reaction occurs, must be allowed.

C. *Temperature*

As a rule, the warmer a disinfectant, the more effective it is. This is based partly on the principle that chemical reactions in general are speeded up by raising the temperature. It is particularly true of coagulation by acids. However, adsorption is decreased as temperature increases.

Alteration of any of these three factors may affect the action of a disinfectant. If it be very cold, time and concentration must be increased. If little time be allowed, then it should be hot and concentrated. If it be very concentrated, then it need not be so warm and will act quickly.

**Some Useful Disinfectants.**—In selecting disinfectants one should give some consideration to their mode of action and the situation in which they are to be used.<sup>8b</sup>

*Tincture of iodine* is the most generally useful for small cuts and abrasions. If the alcohol causes too much pain, the tincture may be diluted with 2 to 4 volumes of water. An aqueous solution containing 1.85 percent of iodine and 2.2 percent of potassium iodide is excellent but has a high surface tension. Anderson and Mallman have shown the value of colloidal iodine for certain purposes.<sup>9</sup> It penetrates well.

*Weak aqueous carbolic acid* (phenol) solution of 0.5 to 1.0 percent has a lower surface tension than water and may be used for washing and dressing in emergencies, but carbolic dressings should never be left in contact with skin, mucous membrane or wounds for more than 30 minutes to an hour unless a *very* dilute solution is used, as they will cause coagulation of the live tissues which may be followed by serious infection (gangrene). *Aqueous 1 to 2 percent crude carbolic solutions* (also *cresol* and similar compounds) make excellent disinfectants for household use, as well as in the barn.

*Cresols* form colloidal (milky) suspensions in water and are therefore especially effective because each colloidal droplet consists of concentrated *cresol*. This is true of any colloidal disinfectant.

*Alcohol* (70 percent) alone or containing *not over* 0.01 percent bichloride of mercury, may be used in similar situations or as a hand wash.

*Hydrogen peroxide* is an oxidizing agent, breaking down readily into water and liberating oxygen. If it can be prevented from thus decomposing too rapidly, it is a fairly effective bactericide, but under most conditions of application its effect is too transitory to be of value because of its instability. It is of value in deep dirty wounds as it prevents growth of anaerobes.

*Solution bichloride of mercury* (1:10,000 aqueous) is useful for general purposes also, but its strong coagulative action must be borne in mind. Like phenol, it will cause irritation and corrosion of skin, mucous membranes and wounds if left too long in contact (hence the old name "corrosive sublimate"). *All* mercury compounds are poisonous and should never be used internally. Its action on bacteria may be reversed by iodine and some other compounds (see page 114).

*Chloride of lime* (0.5 to 1 percent aqueous solution) is excellent for similar purposes and is inexpensive. It depends for its effectiveness on the liberation of free chlorine which, in turn, liberates oxygen from water. The ordinary chloride of lime of commerce is unstable and soon loses most of its free chlorine. Many new compounds which retain their chlorine and liberate it more slowly are more effective and efficient. *Azochloramid* is one of these; *dichloramine toluol* is another. The odor of chlorine may be objectionable.

In addition to the above, there are many new proprietary compounds on the market based on various of the principles given above. New patented chlorine products and dyes, phenol derivatives, etc., are designed for various uses. Specific applications and

directions for use generally accompany packages of these proprietary preparations.

*Sulfanilamide and its derivatives* are now widely used for disinfection of wounds. Packages of these substances are given to soldiers who dust the powder on open wounds. For such purposes the disinfectant should be *sterilized*. This seems contradictory but these powders, being dry, do not act till they come into contact with moist tissues. Some of the dry powders have been found to contain live spores of pathogenic organisms which should be killed before the powder is introduced into wounds.<sup>10</sup> Prompt use of the drug prevents growth, especially of streptococci, thus preventing "blood poisoning," and it is also said to prevent gangrene. Sulfathiazole is preferable for such use, as it does not "cake." These sulfonamid drugs must not be taken by mouth without the supervision of a physician, as they are poisonous.

*Penicillin* is discussed farther on in this chapter.

**Relative Strength and Value of Disinfectants.**—One often hears a disinfectant spoken of as "strong" or "weak" or "mild." These terms are inexact and convey different meanings to different people. To one they mean a "disinfectant odor;" to another, pain on application to a scratch; to still another, corrosive action; to another a pretty color. Rarely does the untrained person think of disinfectants in terms of *bactericidal* strength or toxicity. He has to judge them solely by objective properties. Actually, the value of any substance as a disinfectant depends on a number of factors, important among which are its effect on various materials (discoloration, corrosion, irritation of tissues or toxicity if for use in contact with body tissues), surface tension, cost, and effectiveness or bactericidal action under the conditions surrounding its use. Not all disinfectants are equally effective, and some of them are more effective against some bacteria than against others.

The effectiveness of many disinfectants may be estimated by mixing them with cultures of certain bacteria and measuring the time required for the substance to kill the organisms. If this is done under carefully standardized conditions, *i.e.*, using a constant quantity of culture medium of a stated composition, a fixed temperature, and a suspension of measured numbers of bacteria, the results will be quite accurate and reproducible. Ordinarily, the rate at which 5 cc. of a dilution of a given disinfectant kills all the cells in a small drop (.1 cc.) of 24-hour-old broth culture of *Escherichia typhosa* at 20° C. is compared with the effectiveness of solutions of chemically pure phenol under exactly similar circum-

stances. The effective dilution of phenol under these conditions is stated as unity and the value of other substances rated accordingly.

**The Phenol Coefficient.**—For example, a series of ten sterile test tubes may be set up in a row. In each of the first three are placed 5 cc. of aqueous dilutions of pure phenol in concentrations of 1:90, 1:100 and 1:110. In the remaining seven tubes are placed dilutions of the “unknown,” ranging, let us say, from 1:350 to 1:650. The temperature is adjusted to 20° C. by means of immersion in a constant-temperature bath. Each tube receives 0.1 cc. of the standard broth culture of *E. typhosa*, an interval of exactly 30 seconds elapsing after the inoculation of one tube and before inoculating the next.

Exactly 5 minutes after inoculating the first tube a standard loopful of the fluid is withdrawn and immediately transferred to 10 cc. of sterile broth. This is repeated exactly 30 seconds later with the second tube, and so on till each tube has been tested for surviving organisms at 5-, 10- and 15-minute intervals. The broth subcultures are incubated 48 hours and then readings are made. If growth occurs in any of these subculture tubes, it indicates that live cells of *E. typhosa* still persisted in the disinfectant dilution from which that subculture was made.

The results are tabulated as follows:

		5 Minute Subcultures	10 Minute Subcultures	15 Minute Subcultures
Phenol dilutions	90	—*	—	—
	100	+*	—	—
	110	+	+	+
“Unknown” dilutions	350	—	—	—
	400	+	—	—
	450	+	—	—
	500	+	+	—
	550	+	+	—
	600	+	+	+
	650	+	+	+

\* + = growth; — = no growth.



In this experiment, the 1:90 dilution of phenol killed all the *E. typhosa* cells in 5 minutes as compared with the unknown which did the same in a dilution of 1:350. The ratio of the latter to the former is  $\frac{350}{90}$ , or about 3.9. The ratios at the 10- and 15-minute intervals are found to be 4.5 and 5.5, respectively. Their average, 4.6, approximates the "phenol coefficient" of the disinfectant under investigation.

The determination of phenol coefficients requires a high degree of skill. The result itself has very definite limitations. For example, a disinfectant dissolved in distilled water may have a phenol coefficient as high as 50. However, it may be wholly ineffective if applied in the blood, or used in contact with organic matter such as pus, saliva, feces, milk, etc., as these combine with the disinfectant and remove it from the bacteria. Further, it may have a coefficient of only 2 or 3 when tested against some other organisms, such as staphylococci. When a substance is said to have a certain phenol coefficient, the limitations of the method must be kept in mind.

In order to overcome some of these difficulties the Food and Drug Administration Laboratories make tests of commercial disinfectants with cultures of other organisms and under circumstances designed to imitate actual situations. Thus, tests are often conducted in the presence of blood serum. For testing insoluble or oily products, special methods are used which are intended to approach practical conditions of use. Among these are the filter paper methods (wet and dry) and the agar plate (plain and cup) methods. The following partial descriptions of some of these are taken directly from Circular No. 198, U. S. Department of Agriculture.<sup>11, 12</sup>

**"The Wet Filter-Paper Testing Method.**—The wet filter-paper method is a germicidal test rather than a test of inhibitory properties. It is used when the substance to be tested is not soluble or completely miscible with water, or for substances that are to be used in high concentration, such as soaps, tooth pastes, suppositories, dyes, dusting powders, salves, and ointments.

"No. 2 Whatman filter paper is cut into pieces about 0.5 cm. square and sterilized, then impregnated with *Staphylococcus aureus* by immersion in a 24-hour broth culture of the organism. The wet inoculated squares are then placed in the liquid or solid substance to be tested in such a way as to be completely covered and in intimate contact. At the end of 5 minutes, 10 minutes, 15 minutes, 1 hour, or any other desired length of time, the wet papers are re-

moved with a sterilized, stiff, platinum wire bent at a sharp angle to form a hook and placed in 10 cc. of sterile broth. After as much of the disinfectant as possible has been removed (in the case of sticky substances, the needle must be used to aid in freeing the squares of adherent germicide) the squares are retransferred to a fresh tube of sterile broth (10 cc.) and the tubes incubated at 37° C. for 4 hours, when they are observed for evidence of growth.

**“The Agar-Plate Method.**—The agar-plate method is a test for inhibitory properties and is used for substances remaining in



Fig. 58.—Agar-plate method showing good antiseptic inhibitory properties and good diffusion, clear dark zone. The rest of the plate is cloudy with myriads of *Staphylococcus* colonies. (U. S. Depart. of Agriculture, Circular No. 198.)

contact with the body in the absence of serous body fluids, examples of which are salves, dusting powders, creams, plasters, pads, adhesive tape, catgut, and suppositories. The test organism ordinarily used is *Staphylococcus aureus*. The agar is of the same composition as that previously described for carrying stock cultures of the test organism. Strains of staphylococci may vary greatly in susceptibility.<sup>13</sup>

“Fifteen to twenty cc. of agar are melted and cooled to 42°–45° C. To this is added 0.1 cc. of a 24-hour broth culture of the test organ-

ism. The inoculated agar is then poured into a sterile Petri plate and allowed to harden. As soon as the agar has hardened the test substance is placed in intimate contact with the surface of the agar. If a salve, it is first warmed just sufficiently to soften it and thus secure a complete peripheral contact. As a control, warmed sterile petrolatum may be placed on another portion of the plate. The plates are incubated 24–48 hours under porcelain tops at 37° C. and then are examined for evidence of inhibition. If the preparation

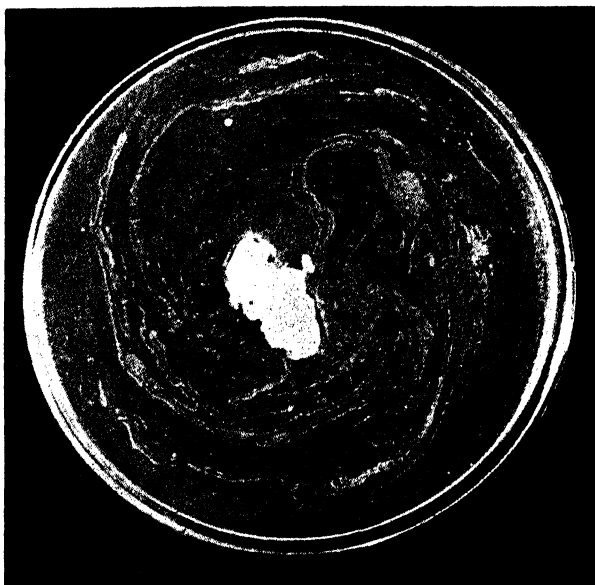


Fig. 59.—Agar-plate method showing no antiseptic properties, or evidence of diffusion. The entire plate is clouded with billions of minute *Staphylococcus* colonies which have grown unhampered by the “antiseptic” being tested. (U. S. Depart. of Agriculture, Circular No. 198.)

is antiseptic or inhibitory, a zone of clear agar will be noted around the place where the substance has been in contact and the width of the zone will indicate the diffusibility of the inhibitory (antiseptic) agent. If there is no inhibition, growth of the test organism will be observed adjacent to and even under the test substance” (Figs. 58 and 59).

*Cup-Plate Method.*—For liquids small circular depressions are made in the agar by inserting glass rods or small vials about 1 cm. in diameter to a depth of about 2 mm. before the agar solidifies

and withdrawing them after it is solid, leaving a little "cup" in the agar to hold the fluid to be tested.

**Toxicity of Germicides.**—One of the great problems in the selection and use of germicides for use in contact with living tissues is the avoidance of excessive toxic effects. Thus, we might use strong carbolic acid on the hands, or bichloride tablets in the stomach, and all bacteria would certainly be killed, but great damage would be done to the tissues by these substances. Certain sulfonamid drugs sometimes have disastrous effects on the blood as well as on bacteria unless the patient is very carefully watched by a physician. On the other hand, penicillin appears to be highly antibacterial yet without much toxic effect on human beings. High-molecular-weight alkyl-dimethyl-benzyl-ammonium chlorides are a class of efficient bactericidal substances with relatively low toxicity for skin, mucosae, etc. They are used in 10 percent aqueous solutions and have low surface tension.

Several methods are available to test the toxicity of germicides, the results sometimes being expressed as toxicity *index*, *i.e.*, ratio of minimal toxic dose to minimal effective germicidal dose.<sup>14</sup> One may determine the toxic dose of a germicide in various ways, for example, by observing the smallest quantity necessary to stop completely the action of phagocytes in a test tube in a given time.<sup>15, 16</sup> Other methods measure the inhibitory or lethal effect of the tested substance on various tissues,<sup>17</sup> while still others measure the respiration quotient of tissue cells in contact with the germicides.<sup>18</sup> One method<sup>19</sup> is based on observing the survival time of chick embryos into which the tested substances are injected. Probably no single test gives a true result, and species of bacteria, as well as of tissue cells, vary greatly in their susceptibility to different germicides. For any disinfectant, the toxicity index will therefore be different for different tissues and bacteria. The conditions of the tests must, of course, be perfectly standardized as to time of exposure to the drugs, temperature, concentration, etc.

### STERILIZATION BY FILTRATION

An exceedingly useful method of freeing fluids from bacteria is by means of filtration. This is seen in its most primitive form when polluted water, seeping through the earth, reappears as spring water, crystal clear, ice-cold and often with very few bacteria. The bacteria and impurities are removed by passage through the soil provided it is not heavily polluted. In the laboratory the same principle is used on a smaller scale, and the procedure is modified

to suit conditions and accomplish a more certain removal of bacteria. Specially graded and purified clays, fine unglazed porcelain, kieselguhr and the like take the place of the earth in the natural process. The clays or porcelain are shaped into convenient, hollow cylindrical forms, closed at one end, and mounted on funnel-shaped stems. They are sterilized by heat before use, so that bacteria in the filter or glassware will not contaminate the filtrate (filtered product). In addition, whereas gravity is the principal force which makes ground water pass downward through the

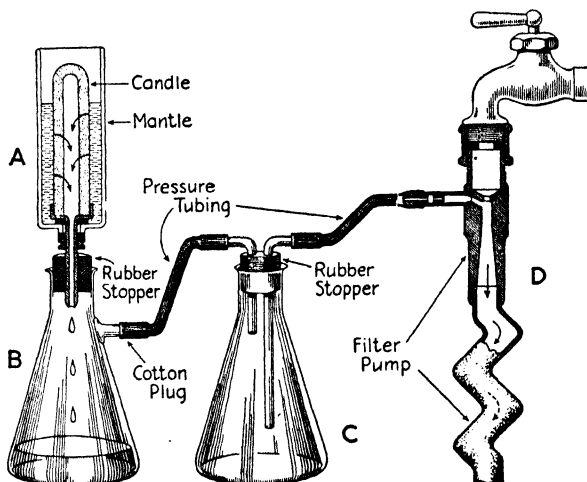


Fig. 60.—Typical filtration apparatus. The hollow “candle” of filtering clay, surrounded by the fluid to be filtered, enclosed in a glass cup or mantle, is seen at *A*. The fluid passes through the clay and is drawn into the flask *B* by suction developed by the pump *D*. The flask *C* is to trap any fluid that might be sucked back from *D* or over from *B*. The cotton plug in the side arm of *B* is to prevent ingress of dust when *B* is disconnected from the tubing. (Belding and Marston, “A Text-book of Medical Bacteriology,” D. Appleton-Century Co., Publishers.)

earth, the laboratory process makes use of atmospheric pressure or the centrifuge to supplement gravity. A discussion of the physics of filtration is given in the section on viruses (see page 703).

Various forms of laboratory apparatus are used, a very common one being shown in Figure 60, and another in Figure 61. A very convenient filter is the Seitz type (Figure 62). A recent development is the Swinney syringe filter, which is the adaptation of a minute Seitz type filter to fit on the end of a hypodermic syringe.

An adaptation of the Seitz principle is seen in the Boerner centrif-

ugal filter. The base of the filter sets into the mouth of a centrifuge tube which receives the contents of the filter cylinder as centrifugal force causes the fluid to pass downward through the tiny disc of

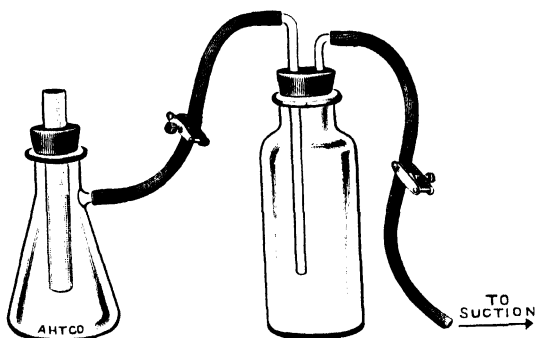


Fig. 61.—Porcelain filter of the Chamberland type.

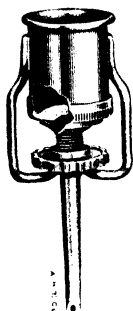


Fig. 62.—Bacteriological filter (Seitz type) adapted for the use of discs of compressed asbestos or paper-pulp as filtering agent. The disc is placed over the wire mesh (which gives it support) in the base of the filter, and the upper portion is then screwed down so as to clamp it tightly in place around the circumference. The stem of the filter is inserted into a suction flask and the fluid to be filtered is poured into the top of the filter.

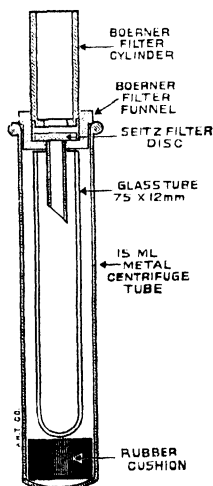


Fig. 63.—Showing Boerner filter in position in 15 ml. metal centrifuge tube, with glass collecting tube.

compressed paper (see Figure 63). An advantage is that suction devices are not needed, another is that quantities of fluid as small as 1 cc. may be filtered.

## BACTERIOSTASIS

Stasis means a condition of immobility or inactivity. Conditions or substances which render bacteria inert or dormant, or simply prevent their multiplication without killing them, are spoken of as bacteriostatic. However, substances or conditions which are bacteriostatic under some conditions may produce death of bacteria if applied for sufficient lengths of time or under conditions of acidity, high temperature, etc. Cold and desiccation are very effective bacteriostatic conditions and are therefore widely used in various ways in the preservation of food-stuffs, textiles, and other materials subject to bacterial spoilage. Their application in the preservation of delicate, unstable biological materials (cultures of bacteria, serums, enzymes, etc.,) has already been discussed (see page 91).

Concentrated solutions of salt, vinegar, sugar, various spices, etc., are effective bacteriostatic agents and are also familiar as food preservatives. Their mode of action is discussed in the section on the effects of environment on bacteria (see page 80). Foods preserved by these means are not necessarily sterile but may become so through storage for long periods. Such foods may at first contain considerable numbers of live bacteria which cannot multiply because of the presence of the bacteriostatic agent used. There are certain bacteria, molds and yeasts, however, which have the ability to multiply freely in quite strong salt and sugar solutions. Some species, as *Lactobacillus* and *Streptococcus lactis*, are common in sauerkraut, ensilage and sour milk, and are called *aciduric* or *acidophilic*. Others, called *halophilic* occur in brines of salt pickle.

**Selective bacteriostasis** is a term applied to any method which creates a stasis of some species but favors the growth of other species. The term is generally used in connection with laboratory methods but is also made use of in industrial processes. A good example is found in the use of the dyes eosin and methylene blue, or basic fuchsin. In bacteriological and medical practice many other aniline dyes also are often used for selective bacteriostasis. Some specific examples will be given later on in this book. The dyes may be mixed with nutrient media designed for the isolation of certain species of bacteria such as, for example, *Escherichia coli* from water. *E. coli* grows readily in the presence of the dyes used, while many other organisms in water are held in check by the dyes.

In attempting the isolation of tubercle bacilli from sputum, various dyes like crystal violet and Congo red are added to the media used, since practically all of the bacteria normally found

in the mouth are inhibited by these substances, while *Mycobacterium tuberculosis* grows perfectly.

As a general rule, all gram-positive bacteria are inhibited by dyes like crystal violet. Just what the relationship between gram-positiveness and sensitivity to these dyes may be is not entirely clear.

Another method of selective bacteriostasis is illustrated when advantage is taken of the fact that certain organisms having bacillary or rodlike morphology are inhibited by the addition of excesses of salt (2 to 20 per cent) to culture media, while many cocci grow under such conditions. This method serves for the isolation of staphylococci from material (urine) containing annoying rod forms (*Proteus*).

Sulfanilamide and related compounds have little bactericidal effect but, like their chemical relatives the dyes, act almost entirely as bacteriostatic agents. They prevent growth of bacteria, especially streptococci, pneumococci and pathogenic *Neisseria* in the body but do little to kill those already there. As soon as the bacteriostatic agents are withheld the bacteria can continue to grow. Therefore, administration of the drugs must continue till the patient is able to drive out the bacteria himself by means of phagocytosis or antibody formation, or both.

Among the most interesting and useful bacteriostatic agents for laboratory purposes is sodium azide ( $\text{NaN}_3$ ). This, unlike aniline dyes, has an inhibitory effect on gram-negative bacteria rather than on gram-positive species. It has been used for facilitating the isolation of fecal streptococci from water (see page 436), for isolating other streptococci and *Erysipelothrix rhusiopathiae*<sup>20</sup> from infectious processes and has considerable value for experimental purposes.<sup>21</sup>

**Possible Mechanisms of Bacteriostasis.**—The mechanism of bacteriostatic action is not fully understood and differs under various circumstances. In the case of extreme cold and desiccation it seems fairly clear that enzyme action and chemical change are stopped because water is immobilized or removed. The action of brines is probably similar to that of desiccation, the brine withdrawing water from the bacterial cell by virtue of its greater osmotic pressure. Dyes like crystal violet may act by coating the bacterial cell with an impervious membrane but this is not at all certain. The possibility that they combine with specific radicles in the cell protein has already been mentioned.

With respect to the means by which the sulfonamid drugs produce bacteriostasis, it appears likely that there is some interference with special enzyme systems, so that multiplication of the



bacteria ceases or is greatly interfered with.<sup>22, 23, 24, 24a</sup> This may be due to the fact that the drug forces certain vital substances out of their proper place in the cell and occupies that place instead, to the detriment of the cell. For example, sulfapyridine and nicotinic acid are chemically similar in some respects. (Fig. 64.) The two substances seem to have similar chemical affinities and to compete with each other for the same position in the synthesis of certain

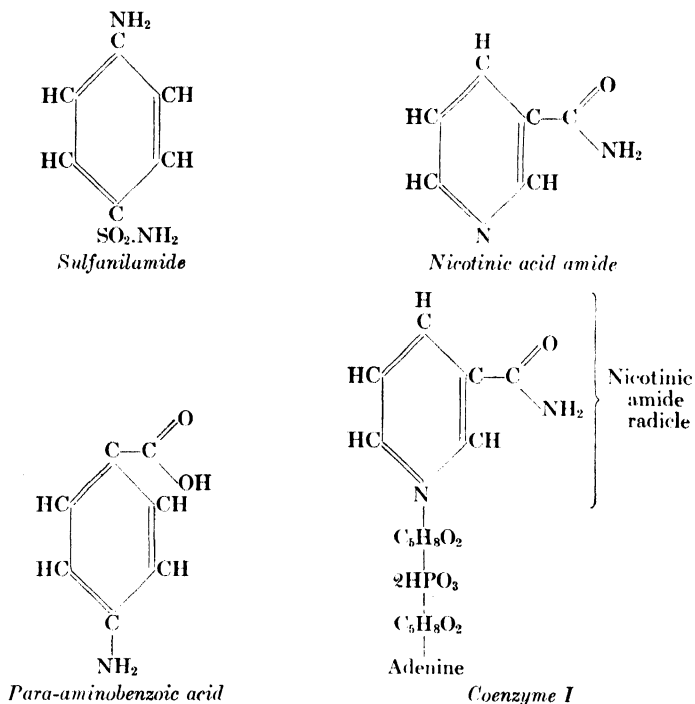


Fig. 64.—Diagrams showing relationships between bacteriostatic and anti-bacteriostatic compounds and the position of nicotinic acid amide in coenzyme I.

enzyme molecules. Either sulfapyridine or nicotinic acid amide can fill a certain position in the structural make-up of coenzyme I or II (see section on enzymes and bacterial respiration, page 363). Nicotinic amide is a normal part of the coenzyme I and II molecules, while sulfapyridine, which can fit into its position, interferes with the function of the coenzymes and prevents growth. *Para-aminobenzoic acid* and sulfanilamide are also chemically related. The former, playing a role in normal cell function analogous to

that of nicotinic acid, has a neutralizing effect upon the bacteriostatic action of the sulfonamid drug. Para-aminobenzoic acid is therefore sometimes added to culture media when attempting to isolate bacteria such as streptococci or gonococci from blood or pus of patients being treated with sulfonamid drugs.

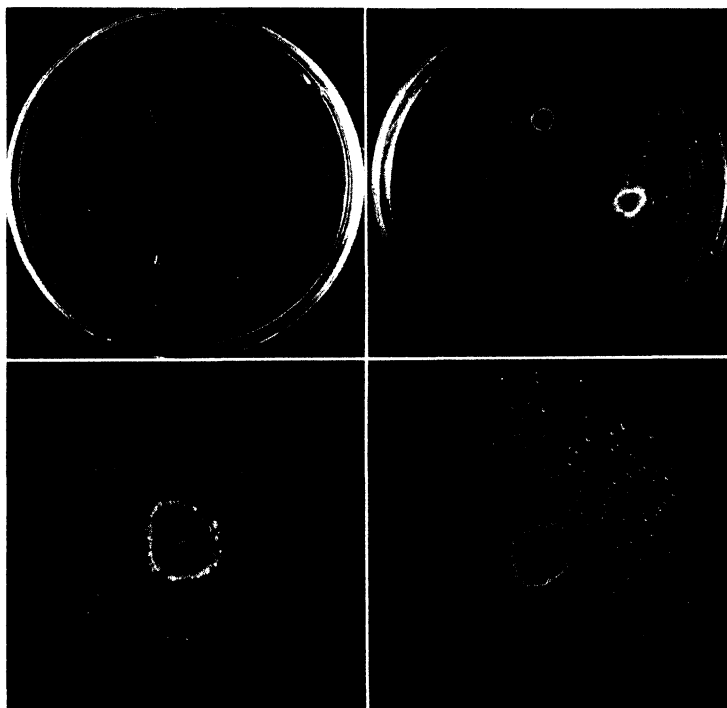


Fig. 64a.—(Upper left.) *Streptococcus pyogenes* as satellite about *Pseudomonas aeruginosa*. (Upper right.) *Shigella sonnei* as satellite about *Staphylococcus aureus* and *Escherichia coli*. (Lower left.) *Streptococcus pyogenes* as satellite about *Staphylococcus aureus*. (Lower right.) *Staphylococcus aureus* as satellite about *Salmonella typhimurim*. (Pike and Foster, Journal of Bacteriology, Vol. 47.)

**Sulfonamid Inhibitors of Bacterial Origin.**—Many bacteria produce substances which inhibit the action of sulfonamid drugs. These inhibitors may be demonstrated on nutrient agar plates containing the drug by showing that the heavy concentrated growth resulting from a “spot inoculation” of a species resistant to the drug permits a localized, satellite-like growth of colonies of a sensitive species of organism dispersed generally over the surface of the plate. Except for colonies appearing as satellites around the spot

inoculation of the inhibitor-producing species, the sensitive organism does not grow at all.<sup>24b</sup> This phenomenon is well illustrated in Fig. 64a. It does not occur on agar without sulfonamid drugs.

It is of interest to note that drug-fast (drug-adapted, see page 97) strains of bacteria have not been found to produce more inhibitor substance after adaptation than before. Another important point is that in mixed infections, as in wounds, one organism, even though harmless in itself, may interfere with therapy against another because it may produce an inhibitor of sulfonamid.

The relationship between sulfonamid inhibitors and the bacteria concerned is roughly analogous to the relationship between penicillin and the enzyme penicillinase produced by some penicillin-resistant bacteria (see page 135), and to the antagonism between certain disinfectants and related substances (see page 114).

Certain other substances can act in the same way as sulfanilamide to displace essential vitamins or growth factors from the enzymes. For example, it has been shown that para-aminobenzenamide has a bacteriostatic effect comparable with that of sulfanilamide. This opens the door to a new cabinet full of "para-aminobenzo" drugs which may have great possibilities like those of the sulfa drugs.<sup>25</sup>

*Action of Sodium Azide.*—Lichtstein and Soule<sup>21</sup> have studied the action, and the mechanisms underlying the effects of sodium azide. *Pseudomonas aeruginosa*, among 41 strains of gram-negative bacteria, was the only species showing even moderate resistance to the drug. The gram-positive aerobic spore-formers (genus *Bacillus*) were also very sensitive, while common staphylococci and diphtheria bacilli were more resistant. Streptococci were highly resistant. Thus we see illustrated again the fact that many drugs, including bacteriostatic substances, while they may show a general tendency to affect a certain type of cell or organism (gram-positive or gram-negative), often exhibit curious inconsistencies and irregularities so that generalization in such matters is never scientifically sound.

It is known that peroxides are products of the aerobic metabolism (see page 364) of many bacteria and that catalase formation is a mechanism protecting bacteria from their own peroxide. Lichtstein and Soule showed that sodium azide markedly inhibits catalase activity under experimental conditions, a fact which probably explains its bacteriostatic action in many species, especially strict aerobes. The same authors showed that sodium azide definitely interfered with oxygen uptake of facultative aerobic species under

aerobic growth conditions. Respiration of these organisms in the presence of the azide seemed to be almost entirely anaerobic. If an oxygen transfer catalyst (pyocyanine in this series of experiments) was present, the "suffocating" effect of sodium azide was largely circumvented by the pyocyanine. A superficial similarity between the actions of sodium azide, sulfonamids and penicillin is thus apparent (see pages 127 and 138).

#### BACTERIOTOXIC SUBSTANCES OF MICROBIAL ORIGIN

Probably everyone has had the experience of finding a "lost" object directly in front of him, or even in his hand, after spending some time hunting for it in obscure places. Similarly, for a long



Fig. 65.—Colonies of *Penicillium notatum* (center), showing surrounding zone of bacterial inhibition. This is the phenomenon noted by Fleming in 1929 which led to his discovery of penicillin. (Merck & Co., "The Story of Penicillin," 1944.)

time scientists sought after species of harmless microorganisms which would kill or prevent the growth of pathogenic ones so that we might "fight fire with fire," so to speak, and prevent or cure disease through the activity of harmless species. Solution of the problem long eluded keen intellects and deep investigations into the problem. Yet the answer lay on the laboratory doorstep, stumbled

over and complained of as a contamination by the bacteriologist on his way to and fro. For years bacteriologists have known that certain environmental bacteria got into their cultures as contaminants and suppressed the growth of the species which they desired to cultivate. They had seen wide, clear spaces devoid of growth, around certain colonies in culture plates containing bacterial and mold growth. The clear zone was of course due to some bacteriotoxic substance given off by the organisms in the colonies thus distinguished. But the phenomenon was so commonplace and attention so fixed on other problems, that the significance of the observations was overlooked until Fleming, in 1929,<sup>26</sup> appreciated its possibilities and acted upon the basis of his idea.

**Penicillin.**—The organism which first attracted Fleming's attention was one of the common molds, *Penicillium notatum* (see Chapter 9), and it occurred to him to experiment with its inhibitory action. He investigated not only the actively growing mycelium on plates (Fig. 65), but the broth in which the mold had been growing (see Frontispiece). By passing the broth cultures through filters he removed the mold filaments and so was able to study the activity of the growth products alone as they occurred in the broth. What he found was that the clarified broth contained a highly potent bacteriotoxic principle, the activity of which was readily demonstrated in contact with sensitive cultures. The broth has been called penicillin, although of course the active bacteriotoxic agent forms only a very small fraction of it.

**Production and Uses.**—The active agent is a waste product of the growing mold. In a culture medium such as Czapek-Dox broth,\* or a "corn steep liquor" medium, often used for the purpose, penicillin is fairly stable at refrigerator temperature if the acidity is not too great. In media like those described acidity first increases and

\* Czapek-Dox medium for penicillin production:

Glucose.....	40	gm.
NaNO <sub>3</sub> .....	3	gm.
KH <sub>2</sub> PO <sub>4</sub> .....	1	gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.5	gm.
KCl.....	0.5	gm.
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.01	gm.
Agar.....	15	gm. (For plating and maintenance media.)
Water.....	1	liter

Mix, tube, autoclave. The agar may be omitted for fluid medium. The addition of 3 to 5 gm. of dried yeast or yeast extract will greatly increase penicillin production. Shallow layers of medium are best as the mold is strictly aerobic. Dark brown sugar has been found more effective than glucose.<sup>32</sup> Zinc is essential.<sup>32a</sup>

then the medium, after five days, becomes alkaline. The penicillin concentration is greatest after about ten days.

Penicillin may be produced on a large scale by three different methods:

1. Surface growth on shallow layers of broth in large flat bottles (Blake bottles).

2. Submerged growth in tanks holding thousands of gallons of medium.

3. Growth on the surface of a finely divided material (usually bran) kept moist with nutrient fluid and aerated in large trays.

In the first method, hundreds of the bottles are inoculated with spore suspensions previously prepared by cultivation of *P. notatum* on Saboraud's agar (see page 172). During the seven to fourteen days of incubation the mold excretes at least three substances of importance: (a) the yellow pigment *chrysogenin*, which must be removed by treatment with charcoal; (b) *penicillin*; (c) *notatin* (this occurs especially if the acidity of the medium is too great).

A substance called *pyrogen* is also found in the culture medium and is undesirable since it causes the patient's temperature to rise when injected intravenously. It is removed with the pigment. Pyrogen is probably present in many culture ingredients and may or may not be produced in large amounts by the mold growth.

After termination of the incubation, during which frequent assays of penicillin strength are made, the fluid is clarified by centrifugation and the penicillin extracted, purified and concentrated by evaporation at low temperature *in vacuo*.

In the submerged-growth process much heavier and more rapid growth and penicillin production are obtained since the mold grows throughout the tanks and not on the surface alone. Constant agitation and aeration are necessary. The mechanical features, including temperature and pH control (essential because of the instability of penicillin), maintenance of sterility, etc., are engineering achievements of the first order.

In the bran-tray method spore suspension is mixed with the moist bran and growth occurs on the surface of the flakes. The penicillin is extracted by washing the bran. The bran extract is then subjected to purification and concentration procedures. In all processes a selected harvest of spores from a potent strain of *P. notatum* is kept as a "seed" stock in order to avoid variations in penicillin yield due to biologic variation of the mold. The spores are dried with sterile soil.

Full details regarding any of the production and purification

methods are not available for publication. However, a rough idea of purification procedures may be gained from the following, which is by now probably largely obsolete because of the rapid progress resulting from constant research in hundreds of large laboratories.

In one process of purification the broth is acidified ( $pH$  2 to 3), and shaken with acetone. The acetone is separated and shaken with buffer solution ( $pH$  7.0). This is then treated with ether and the ether extract passed through an adsorption column containing  $Al(OH)_3$  which adsorbs the penicillin in its upper portion. This

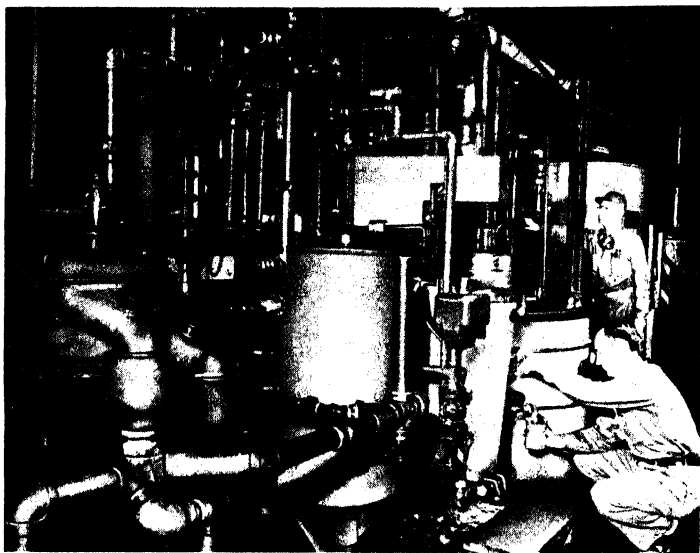


Fig. 66.—Large scale penicillin production is portrayed above. The photo illustrates centrifugal separation during extraction of penicillin from the fermentation broth. (Merck & Co., "The Story of Penicillin," 1944.)

yields its penicillin to ether again, whence it is finally extracted with dilute  $NaOH$  solution ( $pH$  7.2). Many details of technic have been omitted but they are not yet available for publication. A liter of culture may yield a few milligrams of pure crystals. In April 1944, Government experts estimated the nation's output at about 9 pounds of the crystals. The penicillin is very unstable in such solutions, and even in dry form must be kept cold and in a vacuum. Mass production of penicillin is now an accomplished fact although considerable technical difficulties still remain to be overcome (Fig. 66).

The chemistry of penicillin is at present unknown, but Abraham, et al.,<sup>27</sup> describe a tentative empirical formula  $C_{24}H_{32}O_{10}N_2Ba$  to material obtained as a barium salt. Another formula is given as  $C_{14}H_{19}NO_6$ .<sup>27a</sup> T'ung<sup>28</sup> has described a simple and effective means of producing crude penicillin in any laboratory and of concentrating by evaporation in vacuo. The material keeps best if stored on "dry ice." Initial extraction with isoamyl acetate appears to be a practical means of concentration of crude penicillin. The second substance, notatin, produced in cultures of *P. notatum* in addition to penicillin, is also called penatin or penicillin B. This has very striking bactericidal powers.<sup>29</sup> Notatin is active only in the presence of glucose and is not extracted by organic solvents as is penicillin.

Penicillin, when added to culture media or other material, inhibits growth of many species including staphylococci, streptococci, gonococci, meningococci, pneumococci, and diphtheria bacilli, in very high dilutions. Penicillin has also been shown to affect markedly some of the clostridia like *Cl. perfringens* and *Cl. septique*. It is less active, or ineffective, against many of the gram-negative rods such as *E. coli*, *E. typhosa*, *H. influenzae*, and *V. comma*.<sup>30, 31, 32, 33, 33a</sup> Some of these resistant bacteria appear to produce an enzyme, called penicillinase, capable of destroying penicillin. The most interesting results have been obtained in the treatment of syphilis and gonorrhea with penicillin.<sup>34, 35</sup>

*Penicillinase*.—It has been pointed out that many bacteria produce a substance which inhibits the action of sulfonamid drugs (see page 129). Many bacteria, though not necessarily the same species, also produce an enzyme, *penicillinase*,<sup>33b</sup> which destroys penicillin. In the presence of such species the action of penicillin is suppressed or eliminated. In infected wounds, for example, the presence of contaminating species producing penicillinase would greatly interfere with the action of locally applied or intravenously administered penicillin. However, penicillinase is very valuable. Just as it is important to be certain of the sterility of sulfonamid drugs before using them for local application, so it is necessary to determine the sterility of penicillin for similar use or for intravenous injection. Contaminating organisms in penicillin could well escape detection by ordinary cultural methods because the penicillin itself might inhibit their growth until they got into the body tissues. By using purified penicillinase to inhibit penicillin, tests for sterility of the drug are made possible.

There appears to be no relation between the penicillin-resistance



of an organism and its power to produce penicillinase. Cultural conditions such as pH and temperature greatly affect production and activity of penicillinase so that organisms producing it may appear sensitive to penicillin under some conditions and not under others.<sup>35a</sup> Among the species producing penicillinase are *E. coli*, members of the genus *Bacillus* and some species of *Shigella*.<sup>35b</sup>

A highly potent penicillin inactivator has been extracted from certain penicillin-resistant strains of *Staphylococcus aureus*, although *Staphylococcus aureus* is generally very sensitive to penicillin.<sup>36c</sup> Such material may prove valuable for inactivating penicillin in situations already described and in laboratory attempts to cultivate pathogens from patients being treated with penicillin. In the latter field its use would parallel the use of para-aminobenzoic acid in cultures from patients receiving sulfonamid drugs.

The value of penicillin as a bacteriostatic agent in the isolation of such organisms as *H. pertussis* and *H. influenzae* from throat cultures has been demonstrated.<sup>26, 36</sup> About 0.25 cc. of strong solution (broth) is spread over the blood-agar plates used for this purpose and the latter are then streaked with swabs from the throats of patients. Growth of gram-positive secondary throat flora is largely inhibited and the chance of isolating the gram-negative whooping cough organism thereby much increased. Similar applications in other fields of selective bacteriology will doubtless appear in due course.

*Standardization.*—Various methods of standardization of penicillin are used. In one, devised by Foster, growth of a certain strain of *Staphylococcus aureus* in a standardized nutrient broth is measured turbidimetrically, the degree of turbidity depending on the concentration of penicillin.<sup>37</sup> Rammelkamp<sup>37a</sup> has adapted the method to the assay of very small amounts of penicillin in blood, urine, etc. He uses the degree of inhibition of hemolysis by a standardized test strain of hemolytic streptococci as the indicator of penicillin activity. Use of a series of carefully standardized control series of known concentrations allows a computation of the strength of the unknown.

Probably the most generally satisfactory method of standardization is that described by Abraham and Chain,<sup>38</sup> Schmidt and Moyer,<sup>38a</sup> and Foster and Woodruff,<sup>38b</sup> known as the cylinder plate method or the cup assay. The method is based on the principle of the agar-cup method for testing disinfectants as described above. Instead of placing the penicillin solution to be tested in a depression in the agar, however, a small clean cylinder with a per-

fectly flat edge, made of porcelain or of 12-mm. lengths of glass tubing of 8-mm. outside diameter, is sealed by touching it, while slightly heated, to the upper surface of the already-inoculated solid agar and leaving it there. The solution to be tested is placed in the

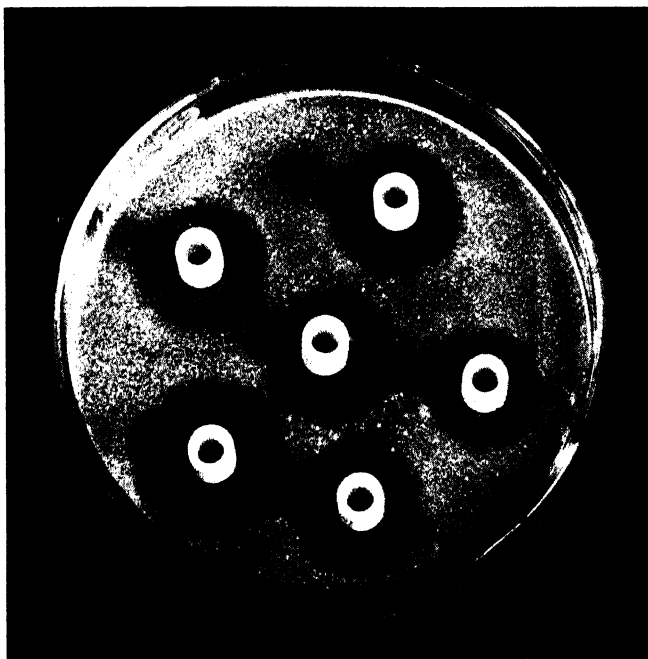


Fig. 67.—Cup plate standardization of penicillin. The culture plate contains a medium, uniformly and heavily seeded with staphylococci or spores of *B. subtilis*. Into each white porcelain cylinder was placed a measured quantity of penicillin-containing extract. Following incubation under standard conditions, growth of bacterial colonies gave the medium the pebbled, gray appearance seen everywhere except around the cylinders. Here, growth has been inhibited by penicillin. Measurement of these zones of inhibition permits standardization of penicillin in Oxford units by comparison with zones produced under identical conditions of test by standard solutions of known unit strength. (Therapeutic Notes, March, 1944, Parke, Davis & Company.)

cylinder (Figs. 67 and 67a). Diffusing into the agar, the penicillin solution reveals its potency by the width of the zone of inhibition. Many minutiae of detail must be considered in making accurate tests. The interested reader is referred to the literature on the subject. Suspensions of *B. subtilis* spores are the most satisfactory test

inoculum.<sup>38a</sup> An agar plate with cylinders in place is shown after incubation (Fig. 67).

Florey has established a unit system of standardization, the unit often being referred to as the *Oxford unit* since it was devised at Oxford University. It is an arbitrary unit based on comparison with a stable solution held as a standard for reference at Oxford. Any preparation adjusted against the original reference standard may serve for assay purposes. The unit is that amount of penicillin which, in 1 cc. of aqueous solution, gives the same degree of inhibition by the cup assay method as the original. This amount of



Fig. 67a.—Filling test cylinder for determination of potency by cup assay of penicillin. (Merck & Co., "The Story of Penicillin," 1944.)

penicillin is the least amount necessary to inhibit completely the growth of the standard test strain of *Staph. aureus* in 50 cc. of a standardized extract broth.

*Properties.*—Among the most valuable properties of penicillin are its solubility in body fluids and its non-toxicity for man and animals even in relatively large doses, whereas its potency is such that a concentration of 0.000001 gm. per cc. of some preparations will exert marked bacteriostatic effects. It may be administered subcutaneously, intravenously or locally. Unfortunately the acidity of gastric juice destroys it unless large amounts of bicarbonate or other alkali are given.<sup>38</sup> Penicillin has the advantage also

that it does not act, as do chemical bactericidal agents like phenol, iodine, bichloride, etc., by nonspecific combinations with *all* proteins so as to produce coagulation or degradation. Such disinfectants are thus rendered inactive in the presence of considerable amounts of pus, blood, etc. Penicillin, on the contrary, probably acts as a specific cell poison for certain species only, possibly by the inactivation of one or more components of their respiratory or hydrogen transport system in a manner analogous to sulfanilamide. The action of penicillin may be very closely associated with the dismutation of pyruvic acid. The inhibition, by penicillin, of pyruvic acid dismutation as normally carried on by living cells, is reversible. The inhibition is removed by washing the penicillin



Fig. 68.—Child with facial and orbital cellulitis, shown at beginning of treatment, ninety-six hours after beginning treatment with penicillin and nine days after treatment was instituted. (Herrell, Proc. Staff. Meet., Mayo Clin. Vol. 18.)

from the cells. This reversible bacteriostatic action is strongly suggestive of the reversible action of bichloride of mercury and dyes. It is of especial significance that penicillin also inhibits the pure enzyme involved in the dismutation of pyruvate.<sup>39</sup> Thus its use in treatment of wounds, blood infections, etc., is feasible since it remains free in the body fluids to act upon the specific bacteria causing the infection.<sup>39a, 40</sup> However, it is quickly excreted by the kidneys.

**Substances Resembling Penicillin.**—Studies by Waksman and others on various molds and fungi have shown that substances having antibacterial properties may be isolated from members of a number of groups of bacteria and molds such as *Ophiobolus*, *Rhizoc-*

tonia, *Fusarium*, *Trichoderma* and *Gliocladium*.<sup>41, 42</sup> Note the absence from the list of the *Phycomycetes*. The field of investigation is new and doubtless other organisms will be added to this list. Studies by White and Hill at Johns Hopkins have shown that bacteriotoxic substances may be obtained from a wide variety of penicillia and aspergilli. From a strain of *Aspergillus flavus* a crystalline substance was obtained which is insoluble in water but which may be extracted with several organic solvents, dilute acid or alkali. Some culture filtrates are active against hemolytic streptococci (Lancefield group A) in dilutions as high as 1:40,000, and against gram-negative rods in lesser degree.<sup>43, 44, 45</sup>

Raistrick, in England, has isolated a penicillin-like substance, *citrinin*, from *Penicillium citrinum*. Although somewhat less potent than penicillin, it has the advantages of being more easily obtained in workable quantities and of being more stable. Data concerning its therapeutic possibilities are still incomplete.<sup>46</sup> Some of the "new" substances may be identical with substances already known.<sup>46a</sup>

**Isolation of Antagonistic Fungi.**—A convenient and simple method of isolating antagonistic microorganisms from soil, manure, etc., consists of mixing heavy, washed suspensions of various susceptible bacteria (*E. coli*, *B. subtilis*, etc.) with sterile 2 percent washed agar containing 2 percent glucose and 0.1 percent of dibasic phosphate, and then inoculating the surface of the plates with dilutions of the material being examined. After 5 days at about 25° C. the desired colonies will appear surrounded by clear zones where the bacteria in the agar have failed to grow. Such antagonistic colonies are fished to pure cultures and studied further for antagonistic properties.

**Tyrothricin and Related Substances.**—In 1931 Avery and Dubos became interested in the destruction of the polysaccharide composing the capsules of type III pneumococci, by an enzyme formed in cultures of certain aerobic, spore-forming bacilli related to *B. subtilis* and found in peat soil (see section on genus *Bacillus*, page 509).<sup>47</sup> It was later shown that purified preparations of the enzyme would protect mice and monkeys against lethal doses of type III pneumococci. The destruction of the capsule of the organisms appears to divest them of their protective coating so that they are readily phagocytized.<sup>48</sup>

Following this line of investigation it was shown in 1939–1942 that the autolysate (culture containing the waste products, enzymes and intracellular substances resulting from spontaneous cell disintegration) of several like organisms obtainable from soil,

sewage, etc., were antagonistic not only to pneumococci but to staphylococci and streptococci and all other gram-positive cocci as well. The antibacterial action is demonstrable with a few gammas ( $1 \text{ gamma} = \frac{1}{1,000,000} \text{ gm.}$ ) of material obtained by alcohol extraction of the precipitate formed at pH 4.7, and reprecipitation with 1 percent sodium chloride. The action, like that of penicillin, appears to depend on inhibition of the dehydrogenases or other enzymes of the bacterial cells. Waksman has pointed out a number of other methods of action of bacteriotoxic substances<sup>48a</sup> and the general problem has been discussed by Dubos.<sup>48b</sup> These antibacterial substances are not identical with the polysaccharide-digesting enzymes of Avery and Dubos, but were discovered during researches in the same field of investigation<sup>49, 49a, 50, 51</sup> and are of similar nature.

The active material is endocellular and may be extracted with alcohol from autolyzed cultures. When so obtained it is active against both gram-positive and gram-negative bacteria of numerous species. An alcohol-soluble material of this sort from *Bacillus brevis* is called *tyrothricin* and has been found to consist of a mixture of two crystalline substances called *gramicidin* and *tyrocidin*. Gramicidin is extracted from tyrothricin with a mixture of acetone and ether; tyrocidin is extracted from the alcoholic residue with hydrochloric acid and has been called also "graminic acid."<sup>51</sup>

**Tyrocidin.**—Tyrocidin is active against both gram-positive and gram-negative species in cultures but is of relatively low potency and is exceedingly toxic when tested in animals. In general it is a nonspecific protoplasmic poison and, like such substances, is to a great extent inactivated by any protein so that its use in serum, blood, pus, etc., is hardly feasible. It appears to be lytic for many species of bacteria.

**Gramicidin.**—Gramicidin, on the contrary, is more like penicillin but is also more toxic and must be used in therapeutics with greater caution. It has little or no antagonistic effect on gram-negative rods. It seems to act specifically on bacterial enzyme systems and is not interfered with by the presence of proteins. It is quite hemolytic and therefore unsuitable for intravenous use. Its insolubility in water contributes to this difficulty and limits its field of usefulness to applications where it comes into direct contact with the bacteria (wounds, abscesses, intraperitoneal use, etc.) Both gramicidin and tyrothricin have been found of particular value in treating mastitis in cattle due to certain streptococci.<sup>52</sup>

**Actinomycin, etc.**—In 1940 Waksman and his collaborators investigated the antagonistic action of a number of soil organisms and later described several substances including *actinomycin* and *streptothricin* obtained from actinomycetes; gramicidin and tyrocidin from gram-positive spore-forming bacilli; *pyocyanase* and *pyocyanine* from *Ps. aeruginosa*; and *gliotoxin* and penicillin from molds. Most of them exert their greatest activity in contact with gram-positive species but pyocyanase and pyocyanin are widely antagonistic, like penicillin. Streptothricin is active mainly against gram-negative bacteria. However, as shown by Robinson, Graessle and Smith,<sup>53a</sup> there is not complete regularity in this respect, and some gram-positive organisms are susceptible. Intestinal bacteria like *E. typhosa* and *E. coli* are very sensitive. Streptococci are resistant, but some species of the genus *Bacillus* are very susceptible. Experiments showed that mice could be successfully treated with streptothricin, administered intravenously or subcutaneously following infections with sensitive species of bacteria. Oral administration was ineffective. The drug is soluble and, while more toxic than penicillin, is not too toxic for successful therapeutic use in mice. How it may affect human beings remains to be determined. Body fluids appear to have no effect on it and it may be of value in local treatment of war wounds and burns. The potencies of these various bacteriostatic agents are enormously greater, gram for gram, than any ordinary chemical disinfectant. Most are much more *bacteriostatic* than *bactericidal* in their effect. Most of them are too toxic for use in human therapy.

From a strain of a new species of actinomycetes (*Actinomyces antibioticus*) two compounds, *actinomycin A* and *actinomycin B*, were isolated. The former is chloroform- and alcohol-soluble and slightly soluble in ether and water and is of an intense red color. It is actively bacteriostatic against many gram-positive bacteria in concentrations as low as 1 in 100 million and against some gram-negative species in concentrations of 1 in 5,000 or higher. It is highly toxic in animals by any of the usual routes of administration. Actinomycin B is more actively bactericidal. When suspensions of gram-positive bacteria are inoculated with *A. antibioticus*, the bacteria are dissolved in 24 to 48 hours. Broth filtrates of the organism will dissolve heat-killed gram-negative species quickly, but act more slowly on similar suspensions of gram-positive species. Most live streptococci and pneumococci are resistant to filtrates.<sup>54</sup>

**General Occurrence.**—It would seem that bacteriotoxins are not

limited to microorganisms but may be found in higher plants as well. For example, a crystalline protein has been obtained from wheat flour by Harris and Stuart<sup>55</sup> which exhibits bacteriotoxic properties somewhat like those of gramicidin. It is also very toxic for animals. In view of its antagonism for yeast, it is suggested that it may be identical with a "yeast-poisonous principle" which has long been known by bakers and brewers to occur in wheat flour.

In 1944 Pratt, et al.,<sup>56</sup> described the production of a penicillin-like substance by a common, single-cell alga, *Chlorella vulgaris*. They called the active principle *chlorellin*. This appears to have valuable properties but full details are still to be worked out. The initial work was done at the University of California and Stanford University in collaboration with the Carnegie Institution.

In the same year Tsuchiya, et al.,<sup>57</sup> announced the discovery by Waller and Gisvold, at the University of Minnesota, of a principle from a plant, which has bacteriostatic properties. The plant is not named but the substance is called *nordihydroquiaretic acid*. It is active against *Salmonella* species, *Sarcina* and some other organisms and is effective in sterilization of the skin. Its value for general therapy remains to be developed.

It appears, therefore, that the whole plant kingdom requires investigation from the standpoint of bacteriotoxic substances. Exact chemical, physical and biological investigations of bacteriotoxic substances are very new, but their great importance has attracted general public attention. Merely the surface of the problem has been scratched. Penicillin has attracted particular notice because of its solubility, low toxicity, high potency and wide range of activity. It has become a successful competitor of the almost miraculous sulfonamid drugs. There are many production problems, such as the tendency to loss of potency and the dual nature of *Penicillium notatum* cultures. There appears to be a conidial or C component and an abnormal mycelial (M) type. The M type is undesirable. Special methods are directed toward maintaining the C mutant predominant in cultures for penicillin production.<sup>58</sup> Much more investigation is needed. A student looking for a fertile field for research need seek no further. The work is fascinating, the technic relatively simple and the reward rich.

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1

## CHAPTER 7

## THE CULTIVATION OF MICROORGANISMS

IN ORDER to cultivate any sort of plant or animal one must have a knowledge not only of its nutrient requirements but also of the environmental factors most favorable to its survival and multiplication. The two are, to a great extent, inseparable but for convenience we will discuss them separately, devoting the present chapter to the methods of arranging the most suitable food for bacteria and related forms in the most effective manner. Methods of cultivating molds and yeasts are similar to those used for bacteria (see chapters on Yeasts and Molds).

**Autotrophic, Saprophytic and Parasitic Bacteria.**—The first forms of life are imagined to have had for their metabolism only simple, inorganic materials, which may be all that were available in the primordial oceans and atmosphere. Among these, probably, were carbon dioxide, hydrogen, oxygen, sulfur, iron, water, and simple,

inorganic salts, including phosphorus and ammonium salts, the last named serving as a source of nitrogen. Possibly simple organic substances also existed. From these our primitive ancestors must have built up their complex chemical structure (protein, etc.), obtaining their energy from oxidative processes going on inside them. Organisms having this type of metabolism still exist, widely distributed in the soil and water, and are called *autotrophic* (*auto* = self; *trophic* = nourishing) bacteria.

It seems likely that, later, or, according to some views, at the same time, forms appeared which utilized more complex organic food materials such as the dead bodies or waste products of their fellows and that these forms initiated and still aid in, the decay processes so essential to soil fertility and to the development and continuance of higher life. Such types are spoken of as *saprophytic* bacteria (*sapro* = decay; *phyte* = plant). Most of the bacteria known today are of this type, and decomposing organic matter such as feces, putrefying animal bodies, plant and animal matter in the soil and hay infusions contain large numbers of them.

Probably still later there appeared, either through progressive or regressive stages or both, bacteria which could live not only upon dead and waste organic matter, but which could also metabolize *living* plant and animal tissues. They caused disturbances of the delicate chemical and physical equilibria of the creatures on which they lived. This was disease and often resulted in the death of the invaded creatures. Such organisms are well known today and are called *parasitic* or *pathogenic bacteria* (*patho* = disease; *genic* = producing).

Since both saprophytic and parasitic forms utilize a variety of complex organic carbon compounds as food, they are grouped together under the heading *heterotrophic*, in contrast to autotrophic species which need no organic materials and are, indeed, often injured or killed by organic matter, at least under conditions of artificial cultivation in the laboratory.

Both autotrophic and heterotrophic bacteria must have whatever food they use in a completely soluble or digestible condition, since the bacteria are entirely *holophytic* with respect to food.

#### CULTIVATION OF HETEROTROPHIC BACTERIA

The early microbiologists found "animalcules" (they applied the term "animalcule" to all extremely minute living creatures, whether of a vegetable or animal nature) in all sorts of decom-

posing organic material. Urine, after standing at room temperature for a day or so, was found to teem with these mysterious creatures. Stale mutton broth or vegetable soups also were seen to contain animalcules, many of which we now know were heterotrophic bacteria. As a result of these observations, the practice grew up of using urine and meat or vegetable broths for the laboratory study of bacteria. The same substances are used today. They contain many soluble protein derivatives, minerals, carbohydrates, fats, vitamins and the like. Foods for bacteria are called *media* (singular = *medium*).

Like all living things, bacteria must have water and sources of important elements, especially, C, H, O, N, S, P, Ca, Na, K, and a number of others. These must be in an assimilable form. For many autotrophic species, the simple elements or inorganic compounds of them will serve. For strict heterotrophs, they, especially carbon, must be provided in the form of organic matter.

**Sources of Organic Matter.** *Animal tissues.*—It has been found that by including extracts of flesh as a source of organic matter the nutritive properties of media for heterotrophic bacteria are much improved.

Two sample formulae given below differ in respect to the type of meat extract used. One ("extract broth") utilizes concentrated extracts like Difco or Liebig's "*Extract of beef*." The other, a much "richer" medium, which supports the growth of a much wider variety of bacteria, especially pathogens, requires that freshly ground meat be soaked or *infused* in water for 2 to 24 hours, and the juices squeezed out and used. Broth made with fresh infusions of the latter kind is called "*infusion broth*." The virtue of fresh meat infusion probably lies in the fact that a greater variety of organic substances, including certain vitamins or "growth factors" (see chapters on metabolism), is present, many of them in a colloidal state.

It may be pointed out that meat is not the only useful source of organic matter. Extracts of vegetables of various kinds are often used by those studying various bacteria; others use the flesh or juices of shellfish, while those interested in the bacteria of milk sometimes use whey, skim milk, etc. Eggs are often used also, especially for tubercle and diphtheria bacilli. An infusion of partly digested soybeans has recently been shown by Brewer to be as good as meat infusion for many purposes.

Some media are made by adding bits of kidney, spleen, or other tissues freshly removed from dead animals under aseptic precau-

tions to tubes of broth. The incorporation of small amounts of some solid or jelly-like substance such as ground meat or sand is often advantageous as many bacteria seem to grow best in the crevices of, or in contact with, the surface of such matter, forming little nests or *niduses* there. It may be that oxygen or food substances concentrate at such points by adsorption. In very dilute media, growth may occur at such surfaces and not elsewhere.<sup>1</sup> The wide practice of clarifying fluid media by filtering out all insoluble precipitate, "for appearance's sake," may therefore be unnecessary and even detrimental. For some purposes, however, water-clear media are necessary.

*Peptone*.—Peptone consists of a mixture of the products of partial protein digestion and includes many amino acids, polypeptides and proteoses, as well as other substances which result from the incomplete digestion of protein. The exact composition of peptone is not known, and is probably highly variable; but it is particularly useful for bacteria which cannot attack whole, undigested proteins like egg, or blood, and which cannot utilize simple elements and inorganic materials as do the autotrophic bacteria.

Along with the organic nitrogen in peptone and meat extracts there are associated, of course, oxygen, hydrogen, carbon, sulfur and phosphorus, in organic forms which are available to bacteria, as well as certain vitamin-like factors of unknown composition. Peptone, therefore, is a very useful source of organic matter and is used in both of the formulae to be described.

#### I. *Meat Extract Broth*:

Beef extract.....	3 gm.
NaCl.....	5 gm.
Peptone.....	10 gm.
Water (distilled).....	1000 cc.

The various ingredients are dissolved in the water as it is heated. Boiling is not necessary at this point. As soon as they are dissolved the reaction is adjusted by the method described below and the medium, after boiling, filtration and sterilization is ready for use.

II. *Meat Infusion Broth*.—The formula using infusion of fresh meat is comparable to the above in every way except that, in place of meat extract, 500 grams of freshly ground, lean beef, pork, veal or other meat are soaked or infused in a liter of distilled water in the refrigerator for 2 to 24 hours. The mixture is then heated to 80° C. and the juice filtered off through gauze or squeezed out by

means of a meat press. Sediment is allowed to settle out in tall cylinders. During this time fat also collects on top and is removed. The volume at this point is usually a little over a liter but, if less, can be made up by the addition of water. Not more than about 100 cc. of water should be thus added. Peptone and salt are added as for extract broth and the medium is prepared as described before. Instead of filtration through paper, however, such material is usually passed through a thin towel or a layer of absorbent cotton enfolded in gauze.

Filtration is said to remove certain nutrient substances and should be omitted except when perfectly clear broth is necessary.

These two media form the basis of many cultural procedures and will be referred to repeatedly later.

**The pH of Culture Media.**—The greatest care in details of preparation will, however, be of little avail if strict attention is not given to the acidity or alkalinity of the medium. This factor is of great importance and, while easily controlled, may constitute the difference between success and failure in cultural work. The reaction in terms of pH must therefore be carefully measured. For measuring pH certain dyes are used which take a definite color at given acidities or alkalinities. A dye like phenol red may be selected. This has a canary yellow color at pH 7.0 or below, a slightly orange or pinkish-yellow color at pH 7.4, is definitely red-orange at pH 7.6 and deep magenta at pH 8.0 or above. Many other dyes of various colors indicating ranges of pH from 1 to 14 are available.

**Measuring pH Colorimetrically.**—Five-tenths of a cubic centimeter of a 0.04 percent alcoholic solution of phenol red, added to 5 cc. of freshly prepared broth, usually turns it a canary yellow color. This indicates a neutral or acid reaction but, as phenol red reaches its maximum yellow color at pH 7 (neutral), we have no way of knowing whether the medium is *exactly neutral* or, if acid, how great a degree of acidity the medium really has. We therefore discard the first test, and select a dye which changes color in a more acid range. For this purpose we may select bromcresol purple, having a pH range from 5.2 (canary yellow) to 6.8 (violet). The test is repeated with this dye and we often find that the color of the fresh medium with this indicator is a reddish-brown, indicating a pH of around 6.0, definitely acid.

In order to determine this exactly (if desired) a comparator block (Fig. 69) may be used, following the scheme outlined below. Tube 1 contains 5 cc. of a solution of *known* pH and 0.5 cc. of the selected dye. Tube 2 contains 5 cc. of medium, but no dye. Tube 3



contains 5 cc. of medium with 0.5 cc. of the same dye. Tube 4 contains water.

Different solutions of *known*  $pH$  (with the dye) are substituted for tube 1 till the color seen on looking through opening *A* exactly matches that seen in opening *B*. We can then state that the  $pH$  of the medium is the same as that of the *known* solution in tube 1.

Tube 2 is included in order to superimpose the natural yellowish tint of the fresh medium on the color of the dye itself in tube 1, since tube 3 also contains the color due to the dye plus the natural color of the medium. Tube 4 is not absolutely necessary but is included to eliminate any slight error or color absorption due to

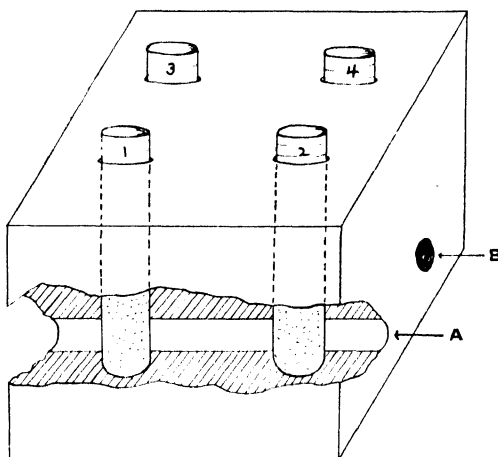


Fig. 69.—Simple apparatus for determining and adjusting  $pH$ . For explanation, see text.

the fact that there are two tubes of fluid seen through opening *A*. The optical equation is thus balanced in *A* and *B*.

There are many modifications of this method. For example, tube 1 with its known  $pH$  color may be replaced, in an appropriate apparatus, by pieces of colored glass.

**Adjustment of the  $pH$ .**—By adding  $N/20$  sodium hydroxide from a buret to tube 3, a drop at a time, we observe that, as each drop is added, the color changes from yellow toward yellow-orange, then to red-orange. Enough hydroxide is finally added so that the color matches that of the  $pH$  7.4 \* standard; or it can be made to match any other standard which we adopt. The amount of  $N/20$  alkali

\* This is a favorable  $pH$  for a wide variety of bacteria.

used to adjust the reaction of the 5 cc. is now noted and the amount necessary to adjust the remaining bulk of the medium is computed. This may be found to be one-fourth of the total volume or more. In order to avoid dilution of the medium by the addition of so large an amount of fluid, we substitute N/1 sodium hydroxide in place of the N/20 alkali, using one-twentieth of the calculated amount. After adding this amount of alkali the reaction of the whole is tested again to verify the correctness of the procedure. The mixture is then boiled for 10 minutes to hasten the formation of precipitates due to the addition of the alkali. Loss of water by evaporation should be compensated for. It may be determined by weighing the vessel of medium before and after boiling. The fluid is centrifuged or passed through a folded filter paper or cotton and gauze to remove any solid matter and may be collected in test tubes or flasks with cotton plugs. It is then sterilized, since, as the early students of spontaneous generation noted, it is a "putrescible fluid" and would soon teem with "animalcules" if not heated thoroughly. The final pH of the finished product should be checked again, as it tends to change somewhat on heating.

**Difficulties with Mixed Cultures.**—The early bacteriologists had to contend with the difficulty that, although the bacteria in their material were often all of one kind, the desired sorts were sometimes mixed with others. Further, as soon as anyone attempted to cultivate the organisms or handle them with instruments, they became still further mixed with bacteria from the instruments, from dust, or from some other source. It was often impossible to distinguish one kind of microorganism from another in such contaminated cultures, since many entirely different kinds of bacteria look exactly alike when viewed with the microscope, even when stained by Gram's method. Pasteur, Koch and many others had the same difficulty. No one could be sure that the chemical or physiological properties he assigned to a given culture of bacteria were really due to one kind of bacterium alone and would remain constant. It was like trying to determine the properties of a salt or element in solutions which contained other salts and elements. It was always a question whether the observed reactions were due to a single kind of organism or to a combination of bacteria growing together in a culture vessel. Such mixed cultures would change their properties as soon as one or the other of the different kinds died off or gained the ascendancy.

One method of separating bacteria, in common use at the time, was to dilute the culture and then put many minute, sep-

arate drops, each by itself, in a separate culture vessel containing sterile broth in the hope that one of the drops would contain only one kind of bacterium. Lister used this method and was one of the very few to succeed with it. It is extremely tedious and inexact, and often fails.

**Origin of Pure Culture Technic.**—Koch had observed the growth of mold and of different sorts of bacteria in isolated masses of various colors on slices of decaying potato in his wife's kitchen. One day, being in an investigative mood, he thrust a slender, sterile, platinum wire into one of the gray bacterial masses and put a bit of it in a little water between two thin slips of glass, under his microscope. Here he saw a great number of bacilli swimming about. Each one of these bacteria looked exactly like the thousands of others in this particular colony. Then Koch examined the bacteria from a yellow colony on the potato, and then those of a red one and of a violet one. The organisms from one colony were all round; from another they all had the appearance of tiny cylinders; from a third they looked like minute, living, spiral springs—but *all the microorganisms in any one colony were always exactly the same.*

Koch appreciated the beautifully simple method which Nature had revealed for him. He realized that every one of the colonies was a *pure culture* of one definite kind of microorganism. When bacteria fell from the air into the various liquid infusions he had been using, the different sorts swam among each other and became mixed. But it was clear that when they fell onto the solid surface of the potato, each one had to stay where it fell. It stuck there and multiplied into millions of its own kind. The mass of growth, or *colony* as it was (and still is) called, was absolutely pure! It was obvious that, by cultivating bacteria on solid food, he could obtain isolated colonies of any kind of bacterium.

**The Use of Gelatin.**—Extending this principle it was but a step to the use of gelatin to prepare a transparent, solid, sticky, nutrient surface on flat pieces of glass. In addition, various nutrient infusions and test substances could be added to the gelatin before it was allowed to "set." Here was a very important advance—a revolutionary advance—one that has been the basis of all our present-day bacteriology. Thenceforth the study and discovery of bacteria in *pure culture* and by relatively exact methods became a matter largely of patience and hard work.

In summer, however, and when held in body-temperature incubators, the gelatin melted and this spoiled everything. It was then no better than the old fluid medium. Being a protein it was

often digested and fluidified by the metabolic processes of the bacteria. Furthermore, when liquid, it spilled off the flat pieces of glass on which it had been allowed to "set," and besides that, particles of dust settled on it with bacteria which contaminated it, obscuring and confusing the results as badly as ever. However, the method was improved, as described in the next paragraph.



Fig. 70.—Modern type of Petri dish.



Fig. 71.—Petri dish with isolated colonies of bacteria from the air ( $\frac{1}{2}$  actual size).

**First Use of Agar-Agar.**—Many students flocked to Koch's laboratory from all over the world to learn his methods. One of these was W. Hesse. To the wife of this man the science of bacteriology is indebted for suggesting, as a substitute for gelatin, the jelly-like substance derived from seaweed, *agar-agar*. Agar is transparent.

colorless, bacteriologically inert, *i.e.*, is not digested or liquefied by most bacteria, melts only at boiling temperature and, once melted, does not set again till about body temperature. Agar has not been improved upon as a solidifying agent for culture media and is in general use for this purpose today.

**Origin of the Petri Plate.**—In order to prevent contamination of the pure cultures by dust, another student in Koch's laboratory, R. J. Petri, suggested the simple expedient of pouring the melted, nutrient agar into flat, shallow dishes, and immediately covering them with a glass cover (Fig. 70). This permitted prolonged examination of the cultures but excluded dust. Such dishes are widely used today and are called Petri plates or Petri dishes (Fig. 71).

The preparation and study of pure cultures then proceeded at a great rate all over the world.

**Preparation of Solid Media.**—Today, procedures almost exactly like those devised by Koch and his associates are in general use in all bacteriological laboratories. The preparation of solid media is comparatively simple. To either of the fluid media prepared as indicated above, gelatin may be added in 10 percent concentration while the fluid is still hot. Since, as Koch found, this liquefies so readily, agar is usually substituted following Frau Hesse's suggestion. Agar is used in 1.5 to 2 percent concentration. These solidifying agents are added before the adjustment of pH.

When using agar, half of the water is withheld from either formula and the agar is melted in this by heating in a steam chamber or over a double boiler. The melted agar is then added to the hot extract-peptone solution or meat infusion.

Other solid media may be prepared by mixing the desired ingredients with eggs or serum, distributing in the desired vessels (usually test tubes), and then heating the vessels till the serum or egg coagulates. A smooth, firm surface results if the heating is carefully done.

**Silica Gels.**—Various methods for the preparation of solid inorganic media by means of silica gels have been described since 1890. A method described by Moore<sup>2</sup> includes various nutrient substances which may be altered at will within certain limits. The solutions given here were devised for halophilic species requiring high saline concentrations (14 percent).

*Solution A*

N/1	H <sub>2</sub> SO <sub>4</sub> .....	50 cc.
N/1	HCl.....	40 cc.
1.6N	H <sub>3</sub> PO <sub>4</sub> .....	10 cc.

Dilute to total acidity of N/5 with sterile mineral solution containing nutrients in 2.5 strength. Peptone and other organic compounds may be used.

*Solution B*

N/1	Na silicate (Bakers 40° Be)	200 cc.
N/1	K silicate (Phila. Quartz Co. 29° Be)	500 cc.
	Conc. $\text{NH}_4\text{OH}$	3 cc.

Dilute to N/5 just before use.

Mix 100 cc. portions of solutions A and B and pour into sterile Petri dishes. Sterile precautions must be used throughout. Such media are used for strictly autotrophic bacteria which will not grow in the presence of organic material.

Improvements in the use of silicic acid jellies for bacteriological use were introduced by Hanks and Weintraub<sup>3</sup> and by Sterges.<sup>4</sup>

**Special Media.**—To any of the broths (or nutrient gelatin or agar or silica gel when in a fluid state) various test and experimental or nutrient substances may be added. Certain carbohydrates may be included to test the fermentative powers of various organisms. Organic esters, blood, glucosides, and many other compounds are put into the medium for a great variety of purposes. The medium is then referred to by the name of the special substance; for instance, "blood-infusion-broth"; "serum-dextrose-extract-agar"; "starch-carrot-whey-agar" and so on.

*Media for Fermentation Tests.*—Under this term are included all media by which the fermenting or hydrolyzing powers of organisms are tested with respect to carbohydrates, organic esters, and also certain glucosides and alcohols. These substances are added to suitable media usually in a concentration of 1 percent before final autoclaving. Substances which are decomposed by heating may be sterilized by passing their aqueous solutions (usually 10 percent) through porcelain, asbestos, or kieselguhr filters, and may then be added under sterile precautions to the medium before use. An indicator, which will change color when and if acid or alkali is produced from the test substance by fermentation, is added before sterilizing the medium,



Fig. 72.—Culture tube showing inverted inner vial for collection of gas resulting from fermentation. Some gas has collected at X.

or after incubating the culture. Bromcresol purple is commonly used. Neither phenol red nor methyl red should be added before incubation as they may prevent growth of some bacteria. Inner inverted vials are often used to trap any gases formed (Fig. 72).

**Blood and Serum Media.**—Blood or serum for media must be obtained under sterile precautions. In small amounts (1 to 50 cc.) blood is conveniently obtained from rabbits, either by incising the marginal veins of the washed, shaved, and disinfected ear or, more simply, by tapping the heart with a needle. The blood may be kept fluid (unless serum is desired) either by defibrinating (shaking for at least 7 minutes with glass beads) or by using 0.2 percent oxalate or 1 percent citrate to prevent clotting. (Use 2.5 cc. of 20 percent aqueous sodium citrate in 50 cc. of blood. The final concentration of citrate is 1.0 percent.) Larger amounts of blood (300 to 500 cc.) may easily be obtained by puncture of the jugular vein of the sheep. The site of the puncture is first carefully shaved and disinfected and a size 16 or 18 trocar, attached by a short length of rubber tube to a suction flask, is inserted.

Citrated whole blood is usually added to media in the proportion of 5 percent. Melt the agar by boiling, cool to 50° C., add the blood, mix by twirling tubes (avoid bubbles) and slant or pour medium before agar solidifies. Suitable controls on the sterility of the blood must be used.

**Synthetic Media.**—This term has been applied to solutions in which all of the ingredients are of known chemical composition, and which can therefore be reproduced at will. Simple solutions of inorganic salts have been known for many years to suffice for the growth of many autotrophic bacteria, and similar solutions with an organic carbon or nitrogen source serve for a wide variety of other bacteria such as *Escherichia coli* and related species. For example Koser developed a medium\* for differentiating fecal *E. coli* from non-fecal *E. freundii* and *Aerobacter* species. Sodium citrate is the only source of carbon in Koser's medium. This salt is readily utilized by the non-fecal organisms while *E. coli* fails to grow.

Within the last few years great progress has been made by the application of knowledge, gained from studies of the nutritive and

**\*Koser's citrate medium:**

Sodium ammonium phosphate (microcosmic salt).....	1.5 gm.
Potassium dihydrogen phosphate.....	1.0 gm.
Magnesium sulfate.....	0.2 gm.
Sodium citrate (crystals).....	2.5-3.0 gm.
Distilled water.....	1000.0 cc.
Sterilize at 15 lbs. for 15 minutes.	

vitamin requirements of bacteria, to the preparation of synthetic media for pathogenic species and others whose needs for organic substances are more complex than the examples given above. A good illustration of this type of complex, organic synthetic medium is one devised for the cultivation of *Corynebacterium diphtheriae* and the production of its toxin. The formula is given below in order to show the nature of such a medium.

*Synthetic medium for C. diphtheriae* (Pappenheimer, Mueller and Cohen<sup>5</sup>).

*Solution A:*

glycine.....	.5 gm
valine.....	1.0 gm.
leucine.....	.5 gm.
glutamic acid.....	5.0 gm.
methionine.....	.2 gm.
tyrosine.....	.1 gm.
NaCl.....	5.0 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	2.0 gm.
Water.....	500.0 cc.

*Solution B:*—(add to A)

cystine..... .2 gm. (in 30 percent HCl, minimal quantity to dissolve).

*Addition 1:*—(Add to A after adding B)

pimelic acid.....	1 mgm
beta alanine.....	1 mgm.
nicotinic acid.....	2 mgm.

Adjust to pH 7.8.

*Solution C:*—(add 0.3 cc. of this to A, after B and 1 and pH adjustment)

CaCl <sub>2</sub> .....	33 gm
H <sub>2</sub> O.....	100 cc.

Boil gently 10 minutes. Filter through paper.

*Addition 2:*—(Add to the above)

MgSO <sub>4</sub> .7H <sub>2</sub> O.....	.3 gm.
CuSO <sub>4</sub> .5H <sub>2</sub> O.....	5.0 mgm.
tryptophane.....	100 mgm.
water to make total volume of 1000 cc.	

Dispense in flasks and autoclave.

Just before inoculation, to each 100 cc. add aseptically .2 cc. of

*Solution D:*

sodium lactate (Merck U.S.P.).....	37 cc.
glucose C.P.....	7.5 gm.
maltose (purified).....	15.0 gm
CaCl <sub>2</sub> .....	0.3 gm.
H <sub>2</sub> O to make.....	100 cc.

Autoclave.



The advantages of synthetic media are that they are exactly reproducible, and in some instances much less expensive and troublesome to prepare than meat infusion and peptone media. Also, they do not contain proteins and therefore have no antigenic properties when injected into man or animals. By virtue of these properties they lend themselves well to exact experimental research and to medical and commercial uses. (See sections on vitamin assay, pages 598, 599.) The great difficulty is that a solution suitable for one species is usually not suitable for another, and it is often difficult to determine the exact requirements for a given species.

An advance toward simplification of procedure, but one that does not avoid the use of complex mixtures of unknown composition (in this case tomato juice), is seen in a formula advanced by Kligler and Grossowicz<sup>6</sup> for the cultivation of pathogenic organisms. The use of meat is avoided.

Solution A:

Peptone.....	5	gm.
NaCl.....	5	gm.
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O.....	2.5	gm.
KH <sub>2</sub> PO <sub>4</sub> .....	0.35	gm.
MgCl <sub>2</sub> .6H <sub>2</sub> O.....	0.3	gm.
H <sub>2</sub> O.....	1000	cc.

Solution B:

Tomato juice.....	40-60	cc.
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Solution A is sterilized as usual. The tomato juice (from fresh, ripe fruit) is filtered through paper, adjusted to pH 7.0 and sterilized by filtration through a porcelain or asbestos filter. It is added to solution A with aseptic precautions. This medium may be used for most ordinary purposes as a substitute for the infusion media previously described. The tomato juice is used as a source of vitamins as well as other unidentified substances which most pathogens require. The necessary aseptic precautions may be troublesome in dealing with large quantities of the medium.

### CULTIVATION OF AUTOTROPHIC BACTERIA

The complex organic mixtures referred to above serve for the growth of nearly all heterotrophic bacteria, certain specific modifications being made in the cultivation of very delicate and fastidious organisms such as the gonococcus and whooping-cough bacillus. For autotrophic forms, the substances used are entirely inorganic, although each genus or family has certain very specific requirements, especially in regard to the substance which they use as a

source of energy. It is impossible to give generally useful formulae which will support growth of all autotrophs, but one or two examples may be cited which will illustrate typical inorganic synthetic media. Thus, for the growth of certain sulfur-oxidizing soil autotrophs (*Thiobacillus thio-oxidans*), a solution such as the following will serve:

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0.2 gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	0.5 gm.
KH <sub>2</sub> PO <sub>4</sub> .....	3.0 gm.
CaCl <sub>2</sub> .....	0.25 gm.
Powdered sulfur .....	10.0 gm.
Distilled H <sub>2</sub> O .....	1000 cc.

For other species, especially those related to soil fertility (*Nitrobacter*), all that is necessary is to prepare a solution like the following:

NaNO <sub>2</sub> .....	1.0 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	0.5 gm.
MgSO <sub>4</sub> .....	0.3 gm.
NaCl .....	0.5 gm.
Na <sub>2</sub> CO <sub>3</sub> (anhydride) .....	1.0 gm.
FeSO <sub>4</sub> .....	0.4 gm.
Distilled H <sub>2</sub> O .....	1000 cc.

The reaction should be about pH 7.2

Such media may be solidified with agar or silica gel, the choice depending on the organisms. Many require the addition of cellulose or glucose since they cannot utilize any other source of carbon. Some are inhibited by organic substances such as agar.

## USE OF LIVING CELLS FOR CULTIVATION OF MICROORGANISMS

**In-Vitro Live-Tissue Cultures.**—Practically all species of bacteria, with a few exceptions among the Spirochaetales, Chlamydo-bacteriales and Thiobacteriales, are known to be cultivable upon lifeless artificial media, *i.e.*, upon material devoid of *living* cells,\* although some, mainly pathogens, require dead tissue or body fluids such as blood serum or ascitic fluid for optimum growth. Many will grow also upon living tissue and in so doing may cause disease, but none are *restricted* to live tissue. This is one of the properties which distinguish bacteria from the viruses and rickettsiae. The last two forms are *obligate parasites*, by which is meant

\* Erythrocytes may be regarded as lifeless since they carry on no known metabolic processes in bacteriological media.

that they cannot multiply outside of, or in the absence of intimate contact with living cells.

One of the simplest and most effective methods of cultivating microorganisms, especially viruses, in living cells *in vitro* was devised by Rivers in 1931 and involves what are generally referred to as "tissue cultures."<sup>7</sup> Many modifications of Rivers' method have been used, but in general such cultures consist of 3 main parts: (1) The suspending fluid, usually Tyrode's solution,\* which furnishes mineral nutrients and glucose and to which, for some viruses such as that of yellow fever, 10 percent serum may be added to supply protective colloids and other materials: (2) The live tissue cells, which may be chick embryo, mouse embryo, skin, liver, testis, kidney or whatever is desired. This material is obtained aseptically and perfectly fresh, minced with sterile scissors in sterile dishes and added to 10 cc. of the Tyrode's solution in 25 cc. flasks, about 0.2 gm. of the minced tissue being used for each flask. (3) The inoculum, which is added in various forms: Tyrode's fluid from another tissue culture, infected blood, bits of infected tissue, etc. The flasks are closed to prevent evaporation and are incubated in the usual manner.

In such cultures, although some microorganisms will not multiply without the living tissue cells, the suspending cell-free fluid is often rich in the organisms. Whether they appear there through rupture of microorganism-containing tissue cells or by multiplication on the exterior surfaces of the cells cannot be stated with certainty. Possibly both mechanisms operate in such instances.

**Cultivation in Chick Embryos.**—One of the great mysteries of biology concerns the susceptibility of embryonic chicks to many diseases, bacterial, rickettsial and virus, to which the mature chick, at the moment of hatching, becomes wholly and solidly immune. What change occurs in the chick at the time of maturation and hatching which makes its tissues entirely insusceptible to organisms which, a few hours or days previous to hatching, would have multiplied unimpeded within the embryo chick or its fluids and membranes, is not known. We may suppose that a complete alteration in metabolic processes takes place immediately on hatching. For example, marked change in oxidation and reduction

\* Tyrode's solution: (Compare with media used for autotrophic bacteria)

NaCl.....	8 gm.	NaH <sub>2</sub> PO <sub>4</sub> .....	0.05 gm.
KCl.....	0.2 gm	NaHCO <sub>3</sub> .....	1.0 gm.
CaCl <sub>2</sub> .....	0.2 gm.	Glucose.....	1.0 gm.
MgCl <sub>2</sub> .....	0.1 gm.	H <sub>2</sub> O to.....	1000 cc.

must occur with the beginning of lung function, and there is a definite change in body temperature. Exactly how these affect the resistance of the chick to the invasion of microorganisms is, however, unexplained. The important fact remains that the fluids, membranes, yolk sac, and tissues of partly matured chick embryos serve as excellent culture media for a great many microorganisms.

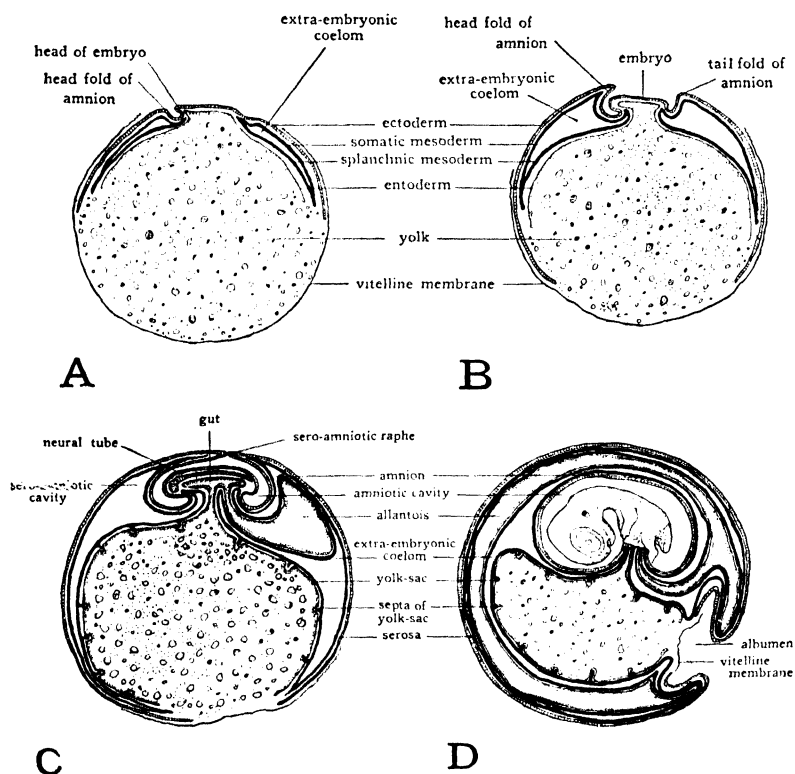


Fig. 73.—Schematic diagrams to show the extra-embryonic membranes of the chick. (D, after Lillie.) The embryo is cut longitudinally. The albumen, shell membranes and shell are not shown; A embryo early in second day of incubation; B embryo early in third day of incubation; C embryo of five days; D embryo of nine days. (Patten, "The Early Embryology of the Chick," The Blakiston Co., publishers.)

**Chick Embryology.**—For the student unfamiliar with the embryology of the chick the following remarks may be helpful. The egg is originally a minute cell in the ovary of the hen and contains a tiny speck of yolk which grows larger in the ovary until it is about 1.25 cm. in diameter. The yolk is nutrient substance. It is enclosed

in a membrane called the vitelline membrane. The cell, with its yolk, is released from the ovary and passes through a tubular structure, the oviduct. Here it receives several coatings of albumen in which it floats (the "white" of the egg). It is fertilized in the upper part of the oviduct. Later it receives the tough shell membrane and, in the uterus, the calcareous shell is added and the egg is then laid. Thus fertilization and the earliest stages of development of the embryo occur before the egg is laid. Under normal conditions of incubation hatching will occur in about 21 days. The early embryonic tissues consist of layers of cells which grow at the expense of the yolk and extend around and later engulf the yolk, lying upon its surface and partly covering it. These tissue layers in great part form the body-cavity walls and organs of the mature chick. The peripheral portions of the embryonic tissue layers become folded off and grow to form the embryonic membranes.

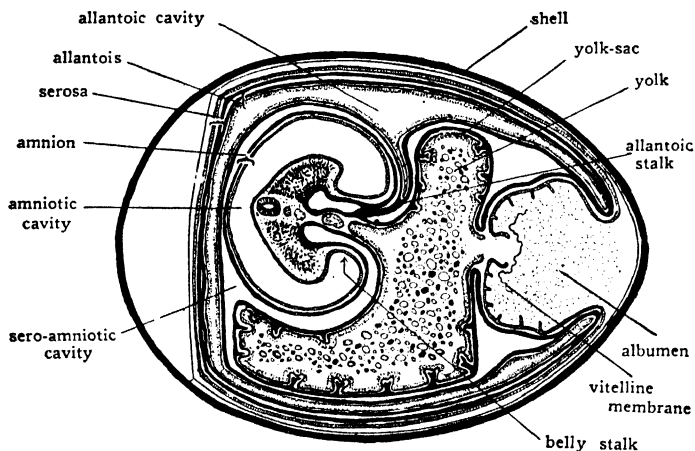


Fig. 74.—Diagram of fourteen-day-old embryo showing membranes and embryo in relation to the shell and air sac. This section is at a right angle to that shown in Fig. 73. (Patten, "The Early Embryology of the Chick," The Blakiston Co., publishers.)

The *chorionic* and *amniotic* membranes form about the embryo from extensions of the above mentioned sheets of cells. These membranes become very extensive and intricately folded and reflected around and over the embryo and several different embryonic cell layers grow together. Afterward the chorion and the walls of a sac developing late in the life of the embryo and called the *allantois* fuse to form the *chorioallantois*, or *chorioallantoic membrane*, which envelops the whole content of the egg. It is this that lies directly below the shell membrane and which is seen as a highly vascular area when the shell is opened. It has an important role in the respiration of the embryo inside the shell. It is left behind when the chick hatches.

Only a course in embryology could make clear to the student the whole mechanism by which the embryonic membranes are formed but the accompanying diagrams (Figs. 73 and 74) may convey some idea of their relation to the embryo proper.

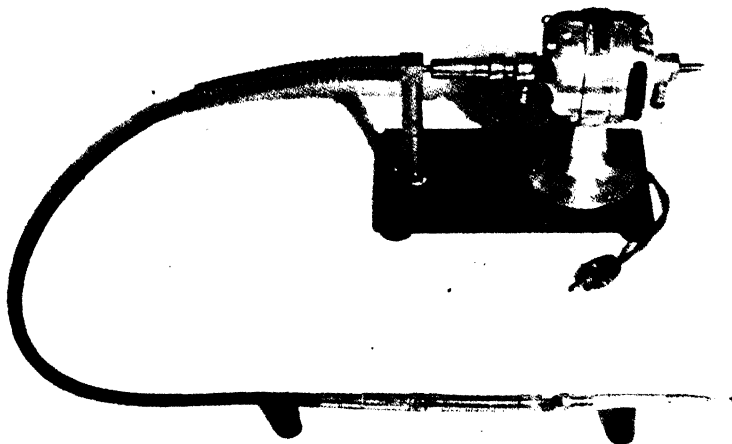


Fig. 75.—Motor, flexible shaft and carborundum disc. (Goodpasture and Buddingh, *Am. J. of Hyg.* Vol. 21.)

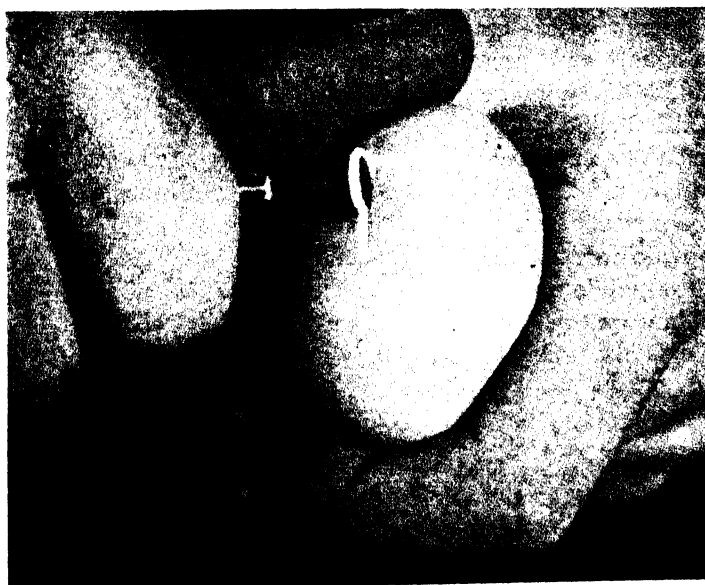


Fig. 76.—Cutting window in shell with rotating carborundum disc. (Goodpasture and Buddingh, *Am. J. of Hyg.* Vol. 21.)



Fig. 77.—Gross appearance of 72-hour lesion of chorio-allantois inoculated with material from variola pustule. Actual size. Fresh preparation unfixed. (Buddingh, *Am. J. of Hyg.*, Vol. 28.)



Fig. 78.—Egg inoculation. Injection of filtered throat washings, from an influenza patient, into the amniotic cavity of a nine day old chick embryo. After twenty-four to forty-eight hours incubation under aseptic conditions, the amniotic fluid and lungs of the embryo are tested for presence of virus by animal passage. (*Therapeutic Notes*, April, 1942, Parke, Davis & Company.)

**Inoculation.**—A very simple and widely used method of cultivating microorganisms on the chorioallantoic membrane of chicks was devised by Goodpasture and his colleagues.<sup>8, 9</sup> Fertile eggs must be

used. They are incubated as for hatching until the embryo is well developed (from 5 to 14 days). To expose the chorioallantois for inoculation, the egg is laid on its side. It may be held in place by spring clips or by pressing it gently into modeling clay ("Plasticine"). The upper surface is then cleaned and disinfected with a weak tincture of iodine and an area about 1 cm. square outlined on the shell. The *shell* is then cut by means of a dentist's fine

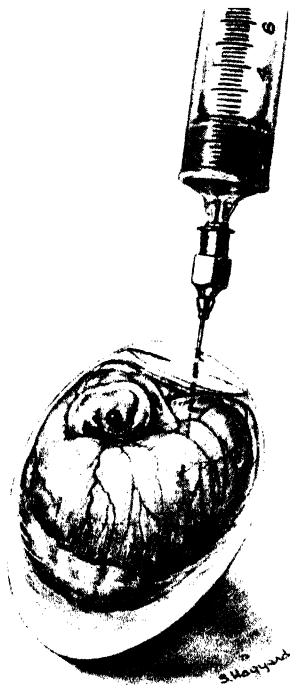


Fig 79.—Inoculation of yolk sac through small hole in end of egg. (Therapeutic Notes, October, 1942, Parke, Davis & Company.)

carborundum disc on a flexible shaft, rotated by a dental motor. (Fig. 75 and 76.) The square of cut shell is then gently lifted off with a sterile forceps, exposing the shell membrane immediately underneath. Before piercing the shell membrane a tiny hole is made in the shell at the large end over the air sac and slight suction is applied by means of a small rubber bulb. The shell *membrane* is then pierced, the chorioallantois drops away from it, and the con-



tents of the egg fill the previously made air sac leaving space under the window cut in the shell. The shell membrane may now be torn off against the cut edge of the shell like paper, leaving the vascular embryonic membranes exposed for inoculation.

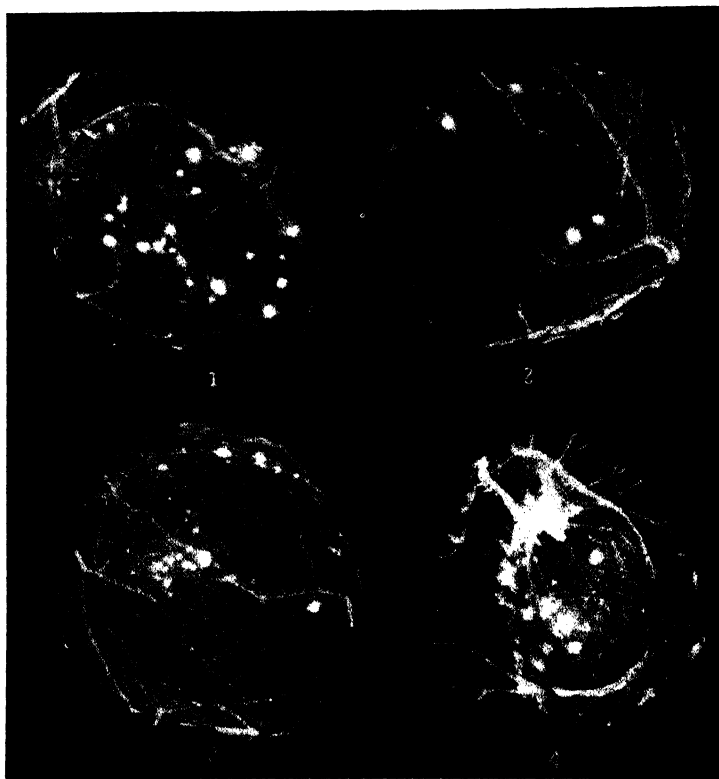


Fig. 80.—Four membranes from a series of vaccinia virus titrations; natural size. 1. Typical satisfactory membrane with 28 specific foci and slight nonspecific edema; 2. Technically perfect membrane with 4 specific foci; 3. Membrane showing a patch of secondary foci near the centre and some probable secondary foci in association with primary foci at edge. This membrane was interpreted as having 6 primary foci; 4. Typical severe nonspecific lesion resulting from gross hemorrhage at the time of inoculation. (Burnet and Faris, *Jour. Bact.*, Vol. 44.)

This is done simply by applying the inoculum with a loop, a dropper, or other gentle means (Fig. 77). The hole in the shell is surrounded by sterile melted vaseline or paraffine and immediately covered with a sterile coverslip of glass, cellophane or gummed

transparent plastic strip. All holes must be stopped by whatever sealing agent is used. The egg may then be incubated as for hatching.

Modifications of this method of inoculation include injection through the membrane directly into the amniotic and chorionic cavities, yolk sac, embryonic tissue, etc. (Fig. 78). For some of these purposes a single tiny hole in the shell is all that is necessary (Fig. 79).

Observations of lesions developing on the chorioallantois may be made through the coverslip and material may be removed for subculture if due care is taken to avoid contamination (Fig. 80).



Fig. 81.—The infected chick embryo and membranes (above) containing virus are removed from the egg under aseptic conditions. (Therapeutic Notes, April, 1942, Parke, Davis & Company.)

The use of eggs for preparing vaccines of several kinds (smallpox, yellow fever, Rocky Mountain spotted fever) has developed into mass production proportions. For example, millions of doses of yellow fever vaccine have been prepared in this manner and used in Brazil and in the U. S. military forces within the last few years.

It may be of interest to note how the yellow fever vaccine is made. The "seed" virus is maintained in tissue cultures as previously described. Selected batches of seed virus may be preserved in a frozen state. For vaccine, eggs with live embryos 7 days old are used. A small hole is drilled in the disinfected shell and the embryo stabbed by a quick thrusting motion with a 27-gauge needle and the fluid from a

seed tissue culture (about 0.05 cc.) is injected. After sealing the hole and incubating the eggs at 37° C. for 3 to 4 days the shells are opened aseptically, the embryos removed (Fig. 81), pooled and weighed and then finely ground up in 9 times their weight of saline-serum mixture.<sup>10</sup> The fluid portion is separated by centrifugation and filtered through Seitz filters and put up in ampoules in a desiccated state. After suitable tests for sterility and potency the vaccine is ready for use. It is highly effective.<sup>11</sup>

Vaccine against Rocky Mountain spotted fever is made by inoculation of rickettsiae into the yolk sac by similar procedures. Here the microorganisms multiply enormously. The vaccine is made of the pooled yolk sac, chorioallantois and embryo, much as is the yellow fever vaccine, except that the rickettsiae are killed with phenol and formalin.<sup>12</sup>

Chick embryos may be used for the study of many different organisms. There are reports on the cultivation of gonococci, meningococci, *Brucella*, *Pasteurella*, *Borrelia*, streptococci, staphylococci, *C. diphtheriae*, *E. typhosa*, fungi, etc. Eggs offer the advantage of economy, availability, constant accessibility for observation of the growth and its effects, excellent opportunity for the study of tissue affinities of various organisms, antitoxins, poisons, and many other problems and phenomena.<sup>13</sup>

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## CHAPTER 8

### YEASTS AND MOLDS

ALTHOUGH neither yeasts nor molds are included in the class Schizomycetes, they belong to the same major division of the vegetable kingdom, namely, the Mycophyta or fungi. They resemble bacteria in many respects and are sometimes very annoying contaminants in the laboratory or preserving kitchen.

Many common species are of great importance in industry, both as the cause of fermentations yielding valuable substances like isopropyl alcohol, acetone, etc., and because of the damage they cause through decay, "mildew," etc. Many are the cause of diseases of plants, animals and man. Several molds, such as *Penicillium* and *Aspergillus*, have assumed enormous importance as the sources of agents exceedingly useful in controlling infections due to bacteria (see section on bacteriotoxic substances, page 131).

Molds and yeasts are often encountered by the bacteriologist incidentally. Also, in descriptions of bacteria, comparisons are often made with yeasts or molds and historical and other references are made to them. The student should therefore have a general knowledge of these organisms. The mass of information concerning them has become very great during the last decade and one who would become thoroughly versed in the matter should make a special study of mycology. In two chapters in this book one can present only a synoptic view of the group.

Like most bacteria and all other fungi, yeasts and molds are devoid of photosynthetic pigments and they grow best in dark or shady places. While, in general, the individual cellular elements composing them are relatively enormous as compared with bacteria, they are nevertheless so minute as to require the use of the microscope for their proper investigation. Yeasts and molds, especially when once isolated on artificial media, may be cultivated

on much the same sort of materials as are used for bacteria and are stained and manipulated in much the same way. Certain media are especially recommended for the cultivation of these organisms, a good illustration of the general type being that of Sabouraud.\* Most of the media for molds and yeasts contain considerable amounts of carbohydrate. The reaction of this type of medium is slightly acid (*pH* about 5.5). Slight acidity seems to favor the growth of many molds and yeasts and inhibits the growth of some kinds of contaminating bacteria.

The yeasts and molds are, as a rule, biochemically versatile and active, metabolizing or attacking a great variety of chemical compounds, and in the soil accomplish much of the decay of organic matter so necessary to life and fertility of the land.

Structurally and physiologically the yeasts and molds are more complex than the bacteria, and may, therefore, be regarded as more highly evolved plants.

**Classification of Yeasts and Molds.**—The differentiation between yeasts and molds is often difficult. Indeed, the transition in form, and manner of reproduction, from one group to another is so gradual that it is difficult to draw sharp lines of demarcation, and existing classifications are like classifications of bacteria, somewhat artificial and arbitrary. For example, there are organisms which not only form, at times, well-developed aerial *mycelia* or woolly filaments, like the molds, but also, under other conditions, soft, pasty growths consisting entirely of free, yeastlike cells. Others may produce no conidia (spore-like bodies characteristic of molds) by which they may be recognized (methods of conidia production being different for each genus or species of mold), or the conidia may so closely resemble yeast cells that differentiation is difficult. Some fungi form conidia which are exactly alike, yet produce very different sexual spores.

For present purposes it is sufficient to indicate the main divisions of the group of fungi, leaving finer systematization for more advanced students of the subject. The following table, adapted from a number of different sources, while incomplete in some respects,

**\* Sabouraud's Agar:**

Water.....	1000 cc.
Peptone.....	15 gm.
Glycerol.....	5 gm.
Dextrose (or Maltose).....	20 gm.
Agar.....	20 gm.

Dissolve all ingredients in water. Do not adjust the *pH*. Sterilize by autoclaving.

may prove satisfactory as a working basis for the present discussion:

### GENERAL RELATIONSHIPS OF YEASTS AND MOLDS

#### Division 1. *Ascomycetes* (sac-forming fungi)

Group I.	{	Not characteristically filamentous. Cells oval or elliptical. Sexual or asexual spores (when present) enclosed in sacs called <i>asci</i> .	{	<i>Saccharomyces</i> <i>Schizosaccharomyces</i> <i>Oidium</i> (short filaments). <i>Torula</i> (no spores yet seen). Cup fungi, powdery mildews, morels.
Group II.	{	Characteristically filamentous. <i>Septate</i> . Asexual conidia borne openly on tips of fertile hyphae. Sexual spores borne in sacs called <i>perithecia</i> .	{	<i>Aspergillus glaucus</i> (green mold common on bread) <i>Penicillium citrinum</i> (blue mold common on decayed lemons)

#### Division 2. *Phycomycetes* (aquatic and terrestrial molds or alga-like fungi)

Filamentous, *coenocytic*. Asexual spores enclosed in *sporangia*. Sexual spores are free (*zygospores*).

{ Aquatic forms cause diseases on fish; some are found on aquatic plants. Example: *Saprolegnia*. Common terrestrial forms are *Mucor mucedo* ("manure mold," white) and *Rhizopus nigricans* ("bread mold," black).

#### Division 3. *Basidiomycetes* (sexual spores borne on special club-shaped stalks called *basidia*)

Consist of *septate* filaments which may be single or may be built up into thick bundles or tissue-like masses as in mushrooms. Sexual spores *free*, borne on stalks which are more than mere hyphae, being specially adapted for spore-bearing.

{ Rusts, smuts, mushrooms, tree or bracket fungi, puffballs, coral fungi.

#### Division 4. *Fungi imperfecti*

{ Not assignable to any of the preceding groups. Most fungi imperfecti might belong in group 2 (the filamentous species) of the *Ascomycetes* but no sexual spores or *perithecia* have been demonstrated. *Torulopsis* might be assigned to this group.

{ Many fungi causing potato and bean diseases belong here. Many ringworm fungi and those causing "athlete's foot" also are placed here pending more complete studies.

The table on page 173 gives a synoptic idea of the relationships of the various divisions of the entire class of Eumycetes, which includes yeasts and molds. The Basidiomycetes, although an important group, are not discussed further because they are not closely related to bacteriology. There are many technical terms in this classification wholly unfamiliar to one who has so far encountered only bacteria in his study of microorganisms. However, these terms will become familiar after a more detailed discussion of the various groups, during which the outline serves as a reference point and a guide.

### THE YEASTS

Because they are structurally among the simplest of the true fungi, the yeasts will be discussed first.

The term *yeast* cannot be accurately defined. One generally thinks of unicellular fungi, never forming mycelia, containing small but definite nuclei, usually multiplying asexually by budding and ascospore formation (see paragraph on reproduction of yeasts) but often sexually by zygote formation or otherwise. The chief difficulty with this, as with most definitions of biological phenomena, is that there are many "exceptions." For example, some yeasts form more or less definite mycelia under certain conditions. On the other hand, many filamentous molds form yeast-like cells, especially under conditions of reduced oxygen tension. Some supposed yeasts are merely unicellular phases of higher molds, including the Basidiomycetes. For convenience, however, we may use the crude definition given above as a basis for discussion, bearing in mind that the group as a whole is not clearly differentiated but is linked by many slightly differing transitional forms with other large groups of filamentous fungi.

**Structure of Yeast Cells.**—The cells of yeasts are quite different, in many respects, from bacterial cells. Morphologically, they are oval or ellipsoidal, rarely forming very long filaments. Yeast cells are, on the average, much larger than bacterial cells. Some oval yeast cells may have a volume thousands of times that of *Staphylococcus* cells and a long diameter two to eight times that of a colon bacillus.

Yeast cells possess all the principal structures found in cells of metaphytes. There is a well-differentiated but small nucleus and a thick cell wall, probably composed of a cellulose-like substance. There are also numerous vacuoles containing food or waste substances and there are granules of various kinds, some evidently related to the formation and storage of glycogen, others composed

of volutin. Yeasts sometimes contain large quantities of fat, of which commercial use may be made.

On the other hand, yeasts resemble many bacteria in being unicellular, nonmotile, devoid of chlorophyll and strictly holophytic in nutrition. They may be cultivated on ordinary bacteriological media, stained by the same methods (they are gram-positive) and, in general, manipulated in the same way. Yeasts also undergo rough  $\rightleftharpoons$  smooth and other variations much as do bacteria, and the phenomena are probably closely related.

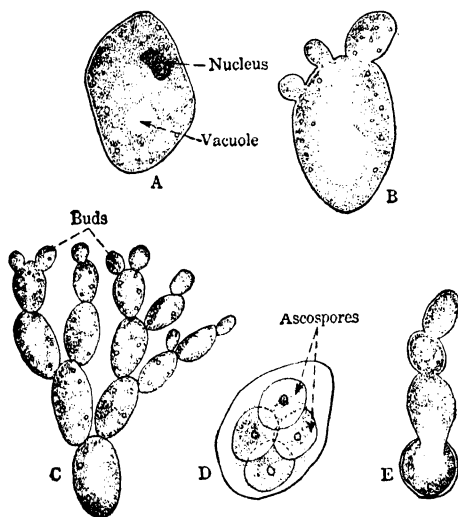


Fig. 82.—Yeast (*Saccharomyces cerevisiae*). A, Single cell highly magnified; B, cell in process of budding; C, chain of cells formed as result of rapid budding and growth; D, formation of ascospores; E, germination of ascospore and the development of new plants by budding. (Redrawn from Curtis. Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc., publishers.)

**Multiplication of Yeasts.**—Yeasts may multiply by one or more of four distinct methods. First, they multiply by a method called "budding" in which large, mature cells divide, each giving rise to one or more daughter cells which are much smaller and which may cling to the parent cell (Fig. 82), often even after the daughter cell has divided. Clumps and often long chains of cells are thus formed (see also "budding" of *Cladothrix polyspora*, etc., page 383).

Second, some species of yeast, in the genus *Schizosaccharomyces*,



divide by equal (binary) fission, much as do the bacteria. This is really a special type of budding.

Third, *ascospores* (spores within a sac or *ascus*) are formed within a single cell when the nucleus undergoes 1, 2 or 3 divisions, without participation of the rest of the cell, forming 2, 4 or 8 ascospores in the sac. There is no involvement of sexes in this process (see Fig. 82). Yeasts belong to the group of *Ascomycetes* or sac-forming fungi because of these spores enclosed in an ascus. The number of ascospores per cell tends to be characteristic of the species. These ascospores are, in many respects, analogous to bacterial spores, being resistant to climatic heat, drought, and other unfavorable environmental conditions. They are not so thermo-resistant as bacterial spores, however, being killed by 60° C. in a short time (see Pasteur's method of preserving beer and wine, Chapter 1). Since they are generally produced in groups of two or more they represent a process of multiplication as well as preservation, thus differing from bacterial spores.

Fourth, the sacs or asci often result from sexual processes in which two cells send out projections which meet and form a copula-



Fig. 83.—Composite diagram of the process of copulation in a species of yeast. Note the ascospores in the fertilized cells. (Redrawn from Guilliermond.)

tion canal and undergo nuclear intermingling, etc.; the whole process is somewhat suggestive of that seen in algae like *Spirogyra* or in certain of the filamentous molds. The resulting cell resembles a zygospore. The nucleus divides to form a number of *ascospores* (Fig. 83).

One interesting point about the reproductive processes of yeast is the probability that in both budding and ascospore formation the nucleus undergoes mitotic division. Haploid\* and diploid\* cells are produced by some species. By placing haploid spores of different species together under favorable circumstances conjugation will occur with the formation of a diploid zygote which germinates into a hybrid yeast. This hybridization of yeasts offers many possibilities to the student of mycology and genetics, especially for commercial purposes.<sup>1, 2, 3</sup> The bacteriologist is forced to content himself with contemplation of mere fission.

\* Haploid means nuclei with half the full number of chromosomes; diploid cells have the full complement of chromosomes.

**Chlamydospores.**—Yeasts are capable of a type of sporulation much like that seen in bacteria. Active growth of the cell ceases, food is stored, the cell acquires a thick protective wall, dehydration takes place, and the resulting dormant cell tides the plant over unfavorable conditions. It is called a *chlamydospore*. It is possible that similar cells are produced by some bacteria, streptococci, for example. Such cells may be seen in yeast cultures or strung like beads along the mycelia of molds such as *Mucor* (Fig. 91). No increase in nuclei occurs as in ascospore formation and the chlamydospore is therefore not reproductive in function. Such bodies are not as heat resistant as bacterial spores.

**Habitat of Yeasts.**—Yeasts are widely distributed in nature. They commonly occur on grapes and other fruits, vegetables, etc., the spores passing the winter in the soil. The kind of wine made from grapes depends to some extent on the varieties of yeasts occurring upon them naturally. Yeasts and torulae (asporogenous, yeastlike fungi) may also be found in dung, soil and milk, and are not infrequently observed in cultures made with swabbings from the throat.

**Activities of Yeasts.**—Yeasts, especially the *Saccharomyces*, are characteristically fermentative organisms, producing chiefly alcohol and carbon dioxide from sugar under anaerobic conditions of growth. Their alcohol-forming power is used in the manufacture of wines and beer and also in the commercial production of alcohol. Varieties especially adapted to each purpose are used. Thus, there are “distillery yeasts,” “top yeasts” for beer, and “bottom yeasts” for beer and wine. Distillery yeasts are the better alcohol producers. Top yeasts produce more foam and alcohol for beer. The yeasts grow in the beer-wort or fruit juice, utilizing the nutrient substances there. Beer-wort agar is commonly used for the cultivation of yeasts.

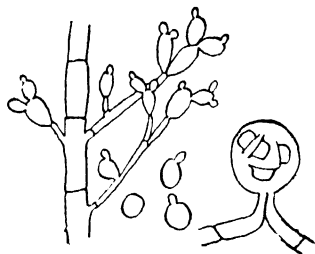
The carbon dioxide-producing power of yeasts is important in baking. Sugar and milk are mixed in the dough. The yeast, utilizing the nutrient substances of the dough for cell synthesis, metabolizes the sugar as a source of energy and bubbles of carbon dioxide are formed which “raise” the bread. Alcohol is also formed. Baking bursts the starch granules, coagulates and dries the matrix so that the bread retains its form, and drives off the alcohol. Some yeasts synthesize many vitamins, including riboflavin.<sup>4</sup> Others require a number of vitamins and this makes them useful in the assay of vitamins by fermentation methods (see page 598).<sup>5, 6</sup>

**Enzymes of Yeasts.**—The fermentations brought about by yeasts

are due to enzymes which are produced in large quantities by the growing yeast cells. Indeed, yeasts are used as commercial sources

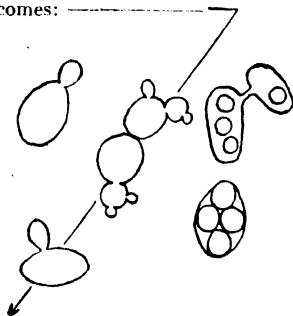
SYSTEMATIC RELATIONSHIPS OF THE YEASTS

ASCOMYCETES	FUNGI IMPERFECTI
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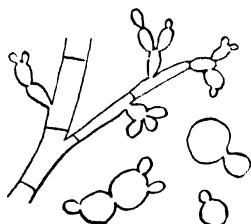


*Endomyces* forms both mycelium and budding single cells. The mycelium forms asci by fusion of contiguous cells. Losing the power to form spores, it becomes: —

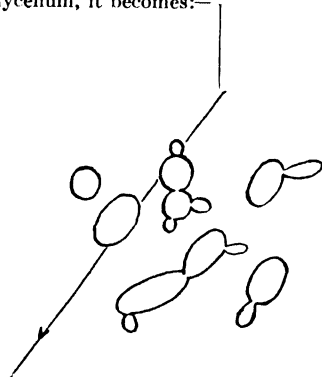
Losing the power to form mycelium, it becomes: —



*Saccharomyces* and related forms; true yeasts, never forming mycelium, existing as single cells reproducing by budding and by spores formed either by the conjugation of neighboring cells or by parthenogenesis. Losing the power to form spores, they become: —



*Monilia*. It forms both mycelium and single budding cells, but fails to form ascospores. Losing the power to form mycelium, it becomes: —



*Torula* and related forms. These are the false yeasts growing as single cells, reproducing by budding, never forming either mycelium or spores.

Fig. 84.—(Reprinted by permission from Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., publishers.)

of some enzymes; these enzymes are then used to bring about fermentations and other changes in the absence of yeasts. The enzyme mixture or complex which produces alcoholic fermentation

## CLASSIFICATION OF YEASTS

## I. Endomycetaceae (Ascospores formed).

## 1. Principally (or largely) mycelial growth.

## (1) Eremascoideae

(Eremascus)

## (2) Saccharomycoidae

## 2. Mycelia with oidia, or oidia alone.

## (1) Endomycoidae

(Endomyces)

## (2) Endomycopseae

(Endomycopsis)

## (3) Nematosporoidae (mycelia and budding yeast cells; ascospores—spindle-shaped)

(Monosporella)

(Nematospora)

(Coccidiascus)

## 3. No mycelia. (Multipolar budding.)

## (1) Saccharomycetaceae

(a) Saccharomyces

(b) Torulaspora

(c) Pichia

(d) Willia

(e) Debaromyces

(f) Schwanniomyces

(g) Schizosaccharomyces.

## (2) Nadsonieae

(a) Saccharomycodes

(b) Hanseniaspora

(c) Nadsonia

Forms intermediate between molds and true yeasts.

(Not included in text discussion.)

Yeasts of commercial importance: some as nuisances, some as valuable aids, to bakers, brewers, distillers, commercial alcohol production, wine makers, etc. Some species also common in soil, dust, etc.

II. Torulaceae or anascosporogenous yeasts (ascospores not formed). Belong to the *Fungi imperfecti*; often called "wild yeasts."

## 1. With conidia.

## (1) Nectaromycetaceae

## 2. Without conidia.

## (1) Torulopsidaceae (no pigments)

## (A) Torulopsidoideae (no pseudomycelium)

Torulopsis (Some pathogens; mostly saprophytes of soil, dust, dung, etc.)

Pityrosporum (Possibly related to diseases of scalp)

Asporomyces

Klockera

Trigonopsis

Schizoblastosporion (various skin lesions)

Mycoderma (common in dust and vinegar)

## (B) Mycotoruloideae (Pseudomycelium formed)

Candida (Various pathogens causing dermatitis, etc.)

Trichosporon (Skin diseases)

## (2) Rhodotorulaceae (carotinoid pigments)

Various pink, red, or salmon-colored varieties, as *Rhodotorula*.

(Often cause spoilage and discoloration of foods.)

of sugar is called *zymase*. Enzymes which hydrolyze disaccharides like maltose and cane sugar (*i.e.*, change the former into 2 molecules of glucose, the latter into dextrose and fructose) are called *maltase* and *invertase*, respectively. These can be extracted from yeast cells by soaking, pressure and filtration.

**Classification of Yeasts.**—A generally satisfactory classification of yeasts is difficult to prepare. Some taxonomists would include with yeasts sporogenous organisms which consist entirely or largely of mycelial growth or regularly produce definite mycelia; others would exclude these and class as yeasts only those which never, or rarely, produce mycelium, and then very little.<sup>7</sup> In the present discussion organisms producing extensive mycelia, although mentioned in the table, are not discussed in detail with the yeasts. The possible evolutionary relationships of four types of yeasts and yeastlike organisms are seen in Figure 84.

We may, for convenience in the present discussion, divide the yeastlike organisms into 2 great groups, Endomycetaceae and Torulaceae, as shown in the table on page 179. The first group includes ascospore-formers; the second group includes species of yeastlike organisms which do not form ascospores. As pointed out above, the mycelium-formers of the first group are named in the table but are not further discussed in the text.

**I. ENDOMYCETACEAE.**—The sacformers, or ascosporogenous yeasts, include the tribes (1) Saccharomyceteae and (2) Nadsonieae, in both of which mycelium formation is reduced to a minimum.

*Saccharomyceteae.*—This tribe includes the genus *Saccharomyces*, which is the largest and most familiar genus and in which are found most of the common yeasts of commerce, such as *S. cerevisiae* and *S. ellipsoideus*. Other genera in the tribe are *Torulaspora*, *Pichia*, *Willia*, *Debaromyces*, *Schwanniomyces* and *Schizosaccharomyces*. All of these produce buds at various parts of the parent cell, *i.e.*, budding is not bipolar. The various genera may be differentiated from one another by fermentation and other biochemical methods and by the shapes of their ascospores or by their modes of germination or modes of formation of ascospores (sexual or asexual). Some are found in beer, others in wine vats, dung, soil, etc.

The *Saccharomyces* are the most common and valuable industrial yeasts. Sexual phenomena are found in this genus,<sup>1</sup> budding and ascospore formation being additional means of multiplication. The cells are oval or elliptical, and oval buds are produced at any part of the cell surfaces (Fig. 85, 8). As mentioned above, their fermentative powers are very useful. There are numerous species

having various special properties of use in industrial processes such as brewing, power-alcohol manufacture, etc.

*Torulaspora* cells form ascospores by simply rounding up and developing a conspicuous fat globule in the center of the cell (Fig. 85, 3). The ascospore in this species consists really of the modified whole cell. Conjugation does not occur although tubes like copulation tubes are formed.

*Pichia* and *Willia* (Fig. 85, 1) are similar genera. Their chief industrial importance is as nuisances in the wine industry since they attack sugar in fruit juices without producing alcohol, and metabolize alcohol in fermenting wine. They give off a very characteristic odor of ethyl acetate and related esters, suggestive of piles of bruised apples fermenting in the summer sun. Their growth in wine vats is in the form of a scum on the surface. *Pichia* is recognizable by its elongated cells, strung together in a distinctive manner, and by its hemispherical or angular spores containing distinct fat globules. *Willia* produces either spores which are hemispherical, having a projecting annular fin like the brim of a derby hat (*W. anomala*) or spores which are flatter with an equatorial projecting fin suggestive of the ring of Saturn (*W. saturna*). The fat globule is distinct.

*Debaromyces* (Fig. 85, 4) form ascospores after conjugation, a single spore to a cell. This species occurs in soil, dust, etc.

The *Schwanniomyces* (Fig. 85, 7) are interesting in that, like *Torulaspora*, under conditions which support spore formation the cells send out elongated processes suggestive of copulation canals, but no fusion occurs. A single ascospore then develops in each cell, the entire cell constituting a spore, as in *Torulaspora* and *Nadsonia*.

The *Schizosaccharomyces* (Fig. 85, 6) differ from all other yeasts in reproducing by binary fission like bacteria. They also reproduce by conjugation of identical cells; eight ascospores result after division of the fused nuclei. Sometimes fusion occurs between a cell and its bud, somewhat as in *Nadsonia*. This process is called heterogamy or heterogamic copulation.

*Nadsonieae*.—The cells of yeasts of the tribe Nadsonieae are characterized by bipolar budding. There are three genera: *Saccharomyces*, *Hanseniaspora* and *Nadsonia*.

*Saccharomyces* are not very common, occurring in decaying organic matter and in soil. The daughter cell is characteristically separated from the parent cell by the formation of a septum between the two, which splits, freeing the daughter cell. The cells are very large.

*Hanseniaspora* are generally easily recognizable by their lemon-shaped cells and rounded spores with a ringlike projection. One or two spores may be formed per ascus. These yeasts are of relatively little industrial importance although they occur in wines and on fruits (Fig. 85, 2).

*Nadsonia* cells (Fig. 85, 9) are large and oval, often showing a small protruberance at one end or buds at both ends. After one of

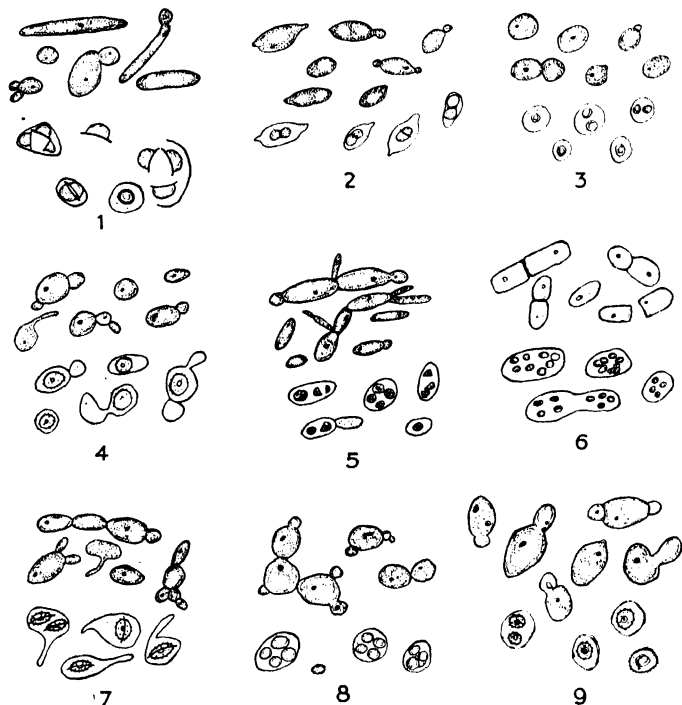


Fig. 85.—Various forms of yeasts, showing vegetative and budding cells and ascospores. 1. *Willia*; 2. *Hanseniaspora*; 3. *Torulaspora*; 4. *Debaromyces*; 5. *Pichia*; 6. *Schizosaccharomyces*; 7. *Schwanniomyces*; 8. *Saccharomyces*; 9. *Nadsonia*.

the daughter cells separates, the nucleus of the other daughter fuses with that of the parent cell. The combined cells fuse to form one large spore of irregular form and containing a large fat globule. The growth of some species, as *N. fulvescens*, is sometimes very mucoid and the fat in older cultures may give a yellow or brownish color.

II. TORULACEAE OR NON-SPOREFORMING YEASTS.—There are

several subdivisions of yeastlike plants forming no ascospores. The largest group, containing several genera, is called *Torulopsidaceae*. These organisms closely resemble the yeasts in most respects except that they have not been observed to produce ascospores by any method. They are, therefore, part of the group known as *Fungi imperfecti*. Their cells are usually more nearly spherical than are the cells of yeasts. Their classification is not complete. The largest and commonest genus is *Torulopsis*, commonly called *torula*. Various kinds of *torula* have been found causing "diseases" of beer and other fermented foods. They are common in soil, water and dust. Some few species are pathogenic, infecting the brain and meninges.

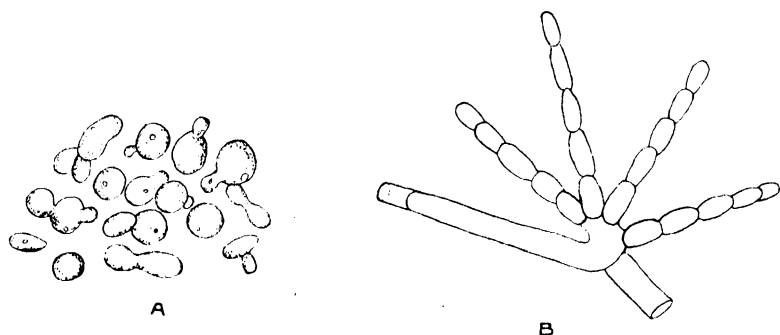


Fig. 86.—Types of oidium cell form and arrangement. *A*, Yeastlike; *B*, moldlike.

Another interesting group, called *Rhodotorulaceae*, produces brightly colored pigments. Various species in this group, particularly red, pink, or salmon-colored varieties, have been described as agents of spoilage in various organic materials and uncooked foods like canned oysters.<sup>8</sup> They are found as nuisances in places where food stuffs are prepared, as in butcher shops and oyster-shucking establishments. They are not pathogenic. Most of the torulas have little fermentative ability and consequently are of little commercial value.

Several pathogenic genera of fungi are classified near the torulae, especially certain ringworm and dermatitis fungi, as indicated in the table.

The genus called *Oidium* tends to form filaments of cells which often elongate to such an extent as to resemble the hyphae (filaments) of molds. The filaments fragment readily into single oval cells called *oidia* and their yeastlike characters then become more apparent (Fig. 86).



**Therapeutic Value of Yeast.**—Most of the yeasts occurring in compressed “yeast cake” are *Saccharomyces* and are of the “top yeast” variety. The cakes consist partly of starch. The yeast cells synthesize several vitamins which are of great value in the maintenance of health.<sup>4, 5, 6</sup> Yeast may therefore be taken *if the diet is otherwise deficient in these vitamins*. The number of cakes eaten must depend to some extent on the availability of other sources of vitamins. Normal foods, in good variety, including eggs, milk, butter, vegetables such as spinach and lettuce, whole cereals and citrus fruits, furnish practically everything offered by yeast, and in a more rational form.



Fig. 87.—Blastomycosis due to *Oidium dermatitidis*. (Courtesy of Dr. Isaac R. Pels, from Andrews, “Diseases of the Skin.”)

### Pathogenic Action of Yeasts.

—A number of diseases, some of them very serious, are caused by certain yeastlike fungi.

*Thrush*, an ulcerative disease of the mouth and throat, is caused by a yeastlike organism variously called *Oidium albicans*, *Saccharomyces albicans* and *Monilia albicans*, the multiplicity of generic names reflecting the difficulty in classifying it exactly. The organism is prone to form moldlike filaments. It may invade the stomach and intestines, possibly causing the ulcerative, diarrhetic disease known as sprue, but more likely acting

only as a secondary invader. Sprue is probably due to dietary deficiencies.

*Blastomycosis* is a severe and often fatal disease in which yeastlike organisms invade the skin and subcutaneous tissues, causing swellings which ulcerate (Fig. 87). The organisms, *Oidium dermatitidis*, sometimes invade the lungs, bones, spleen and other organs.

### REFERENCES

1. Lindegren, C. C., and Lindegren, G. Segregation, Mutation and Copulation in *Saccharomyces Cerevisiae*. Ann. Missouri Bot. Gard., 1943, 30:453.



divisions of the Eumycetes; namely, Ascomycetes (group II), Phycomycetes, and Fungi imperfecti. Peculiarities of structure and methods of reproduction enable us readily to distinguish filamentous Ascomycetes from Phycomycetes and we shall discuss these details first, comparing the two groups in a general way.

*The filaments of molds* may consist of elongated cells arranged end-to-end and separated by walls (*septa*) as in the Ascomycetes or, as in the Phycomycetes, the whole mycelium may consist of one continuous protoplasm-filled tube which is coenocytic (not divided into sections by septa). In mature plants septation or nonseptation is a fairly stable characteristic and serves as an aid in differentiation among molds. The nuclei of molds, within the filaments, are extremely minute, but generally quite definite. In the Ascomycetes each cell contains one nucleus. In the Phycomycetes the nuclei are not separated by definite cell walls except as a result of the formation of spores.

*The cytoplasm* of molds is granular and contains droplets of fat, carbohydrate and nitrogenous material, including volutin. The cell wall of some species probably consists of cellulose; in many others the cell wall seems to be made of a chitin-like substance.

**Reproduction of Molds.**—Portions of the mycelia of molds, when transferred to new media, will usually continue growth much as other plants do, the hyphae continuing to elongate, usually by growth at the ends. After a time *fertile* aerial hyphae are produced which begin the process of multiplication by formation of *asexual* spores (*conidia*). The color of mold colonies, when present, is usually due to the asexual conidia, which are borne in enormous numbers on the fertile hyphae.

Spores formed by processes analogous to *conjugation* or sexual fertilization also occur in some species. For example, in the Ascomycetes, both filamentous and nonfilamentous, two morphologically *different* cells unite to form the sexual spores. The spores thus formed are enclosed in sacs or asci and are called *ascospores* (hence, Ascomycetes).

In the Phycomycetes sacs are not formed, but sexual spores result from the fusion of either morphologically *similar* or *dis-similar* cells. Such spores are called *zygospores* and are free from sacs. Often the differentiated cells which fuse to form zygospores, as in *Rhizopus*, are on two separate plants, which produce the spores wherever hyphae come into contact, suggesting that the plants are of opposite sexes. Neither plant alone produces zygo-

spores. Such plants are said to be *heterothallous*, and their "sexes" are called + or -.<sup>1</sup>

*Fungi imperfecti* are those (chiefly much like the Ascomycetes) in which no spores have been observed. This, however, does not prove their absence and, as Henrici points out, the imperfection about *Fungi imperfecti* is largely in our knowledge of them.<sup>2</sup>

Further details of reproductive methods will be taken up later in connection with descriptions of individual species.

**Microscopic Examination of Molds.**—For the microscopic examination of molds the slide culture is an excellent method. A good

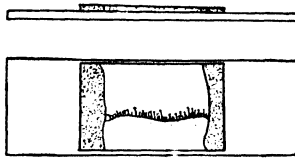


Fig. 88.—Method of growing molds between a slide and cover slip. (Reprinted by permission, from Henrici, "Molds, Yeasts, and Actinomycetes," by John Wiley & Sons, Inc., publishers.)

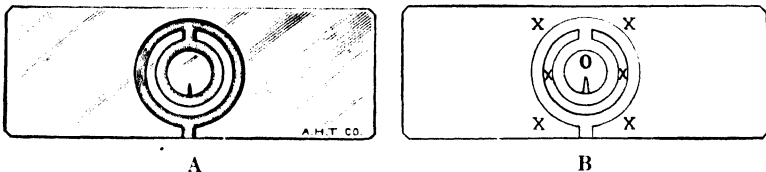


Fig. 89.—Special slide for cultivation of fungi. (Brown.) The inoculated medium is placed upon the stage and covered with a thin cover glass and sealed with oil. Air can reach the culture only through the moats, which delays evaporation of the culture medium and results in minimum danger of contamination. The design of the slide also permits a thin layer of medium so that the aerial and deep growth can be observed under high magnification.

arrangement is to cement a sterile cover slip to an ordinary (previously sterilized) slide with two other bits of glass arranged so that the cover slip is raised about 1 millimeter above the slide. The desired nutrient agar, melted, cooled to about 45° C. and inoculated with spores, is admitted between the slide and the cover slip and the culture incubated (Fig. 88). This permits examination of the growth with fairly high power lenses and in a living, undistorted condition.

A special type of slide-culture vessel has been devised by Brown<sup>3</sup> for the same general purpose. This is shown in Figure 89.

**Method of Use.**—The slide is sterilized by passing it through a flame. If agar is to be used, the slide should be placed on a warm stage or on a hot water bottle. Small droplets of mineral oil are placed at the six points marked "x" in Fig. 89, B. A large loop of the inoculated medium (for example, Sabouraud agar) is placed on the central stage at the spot marked "O". The preparation is then carefully covered with a flamed 25-mm. cover glass which should cause the inoculated medium to spread out in the form of a broken circle on either side of the notch at the edge of the stage, which notch serves as an air inlet. The slide may now be incubated in a Petri dish containing a piece of moist filter paper to prevent drying.

If it is desired to arrest growth of the culture at any stage and to make a permanent preparation, place the slide culture in a desiccator over formaldehyde solution for several hours, and then seal off the exterior air inlet, i.e. short, straight moat extending from edge of slide to outer concentric moat, with paraffin by means of a hot spatula. The culture can be observed at any time under the dry objectives of the microscope. Structures near the cover glass can be observed under the oil immersion objective.

Material from Petri dish cultures may be examined with low power lenses *in situ*, or portions may be teased out on a slide in a drop of lactophenol or mounting fluid\* and examined under a cover slip. Drying produces shrinkage and distortion, while manipulation, such as teasing out, causes fracturing and loss of conidia.

**Habitat of Molds.**—Mold conidia are to be found in all dust, as every housewife knows who has had preserves spoiled by them. They occur on decomposing organic matter like manure piles, "compost" heaps, dead plants and animals. They can thrive especially under conditions where there is too little water for the active growth of yeasts or bacteria. For example, they grow as "mildew" on books, old shoes, clothing and floors in damp weather.

They can grow in fluids having far too high osmotic pressures for yeasts or bacteria and are therefore a very common cause of spoilage in jams, jellies, pickles, and the like.

Physiologically they are, as a group, active enzyme producers, causing very rapid fermentations, proteolyses and other biochemical changes in a great variety of substances. They are of great commercial value in the production of certain enzymes and various organic compounds which are used as foods, flavors and drugs.

Some of them are highly pathogenic. Most molds are readily cultivated on ordinary bacteriological media such as infusion agar; Sabouraud's is very useful. Bread is also an excellent culture medium for many molds.

\* A useful mounting fluid for fungi consists of a mixture of equal parts of glycerine, ammonium hydroxide and ethyl alcohol.

## THE PHYCOMYCETES

The group of Phycomycetes contains two genera of very commonly observed and often very troublesome molds, namely, *Mucor* and *Rhizopus*.

**Genus *Mucor*.**—This genus contains several dozen species, many of them very similar to one another. One of the best known of these is *Mucor mucedo*, a coarse, woolly, white mold seen on piles of rotting manure or other decaying organic matter. It is active in the chemical decomposition of fats, proteins and carbohydrates.

*Mucor mucedo* forms large numbers of submerged hyphae and reproduces asexually by means of *sporangia*, filled, as a rule, with black spores. Each sporangium is borne on a short *sporangiophore* (Fig. 89a, A) (compare *conidiophore* in the filamentous ascomycetes) at the tip of which is

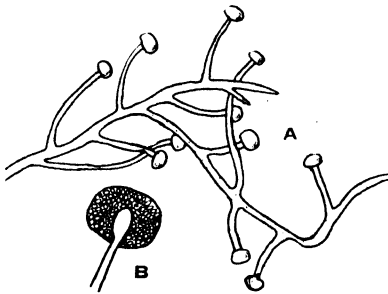


Fig. 89a.—One species of *Mucor*. Mycelium with sporangiophores and sporangia at A; detail of sporangium and columella at B

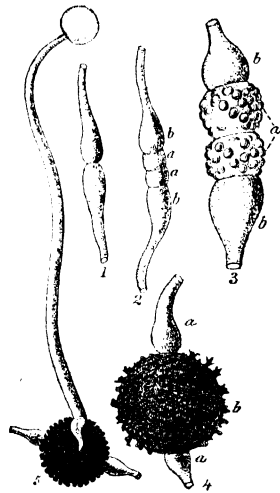


Fig. 90.—Successive stages of zygo-spore formation in *Mucor mucedo*. (From Jordan, after Brefeld.)

an enlarged portion called a *columella* which varies in shape according to the species. The spores are formed in a mass about the columella within the envelope (Fig. 89a, B).

During sexual reproduction the tips of two hyphae touch each other (Fig. 90, 1) and grow into contact, the apical portions of each being segregated by septa and swelling to form larger bodies, the *gametes* or sexual cells (Fig. 90, 2a and 3a). These fuse, resulting in a cell called the *zygospore* (Fig. 90, 4b). This goes into a "resting stage" of greater or less duration and eventually, finding conditions

of moisture, temperature and nutrition favorable, bursts or germinates, sending forth a hypha which may be either fertile or vegetative (Fig. 90, 5). *Zygospore formation is limited to the yeasts and Phycomycetes*. *Mucors* and related genera of molds are common

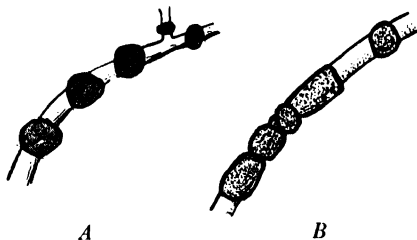


Fig. 91.—Chlamydozoospores. A, *Mucor*; B, *Rhizopus*. About  $\times 400$  (after Brefeld).

contaminants of bacteriological cultures. A few have been found as the cause of disease in man and animals.

*Chlamydozoospores*.—As in the yeasts, cells of *Mucor*, *Rhizopus*, and related species often become rounded, acquire a thickened wall and go into a quiescent state somewhat resistant to drying,

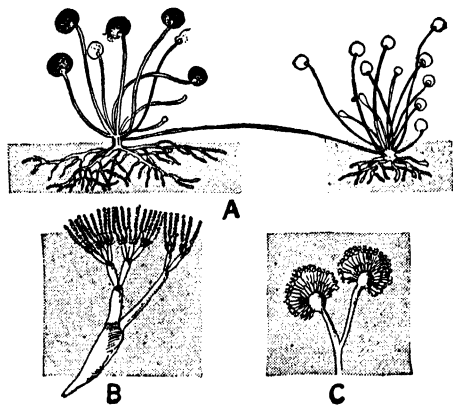


Fig. 92.—Types of mold. A, Species of *Rhizopus* showing stolons and hold-fasts. B, Asexual spores of *Penicillium*. C, Asexual spores of *Aspergillus*. (Hunter and Whitman, "Problems in General Science," American Book Co., publishers.)

sunlight and other mildly unfavorable influences. Such cells are often seen distributed along filaments of the molds (Fig. 91).

**Genus *Rhizopus***.—The molds of this genus are well exemplified by *R. nigricans*, the common, black, bread mold familiar to all who have seen bread after it has stood in a humid place for some

days during the summer. It spreads rapidly because it sends out stolons or runners (Fig. 92, *A*) like some kinds of grass ("crab grass") and strawberry plants. These runners take hold of the substrate by means of "hold-fasts" or rootlike hyphae.

Like *Mucor mucedo* and its allies, *R. nigricans* is an active enzyme-producer and is prominent in bringing about decomposition and spoilage of various fruits, vegetables, and other organic materials.

Molds of the genera *Mucor* and *Rhizopus* are similar in many respects but may be easily differentiated by at least three characters as follows:

	<i>Mucor</i>	<i>Rhizopus</i>
Runners. ....	Absent.	Present.
Sporangiophores. ....	May arise at any point in the mycelium.	Arise only at the "hold-fasts" where the runners touch the substrate.
Columella. ....	Never hemispherical; continuous with the sporangiophore.	Hemispherical; a single, differentiated cell.

### THE FILAMENTOUS ASCOMYCETES

The group of Ascomycetes as a whole contains many widely differing plants, varying from the yeasts, through the filamentous



Fig. 93.—Species of *Aspergillus* showing structure and arrangement of conidiophores, mycelium and perithecia.

woolly molds, to fleshy mushroom-like morels and the cup-fungi which grow on rotting logs. All have in common the formation of asci with ascospores and all are modifications or evolved patterns of the same basic growth design. The yeasts have already been



described. As morels and cup-fungi have no relationship to bacteriology, they will not be discussed here. This leaves some of the common filamentous Ascomycetes for our attention. Two of the best known genera are *Aspergillus* and *Penicillium*. It must be pointed out that, although we discuss sexual processes of *Aspergillus* and *Penicillium* at this point, the sexual structures of most species are unknown and *Aspergillus* and *Penicillium* are therefore important genera in the group of Fungi imperfecti (see page 195).

**Genus *Aspergillus*.**—Like other filamentous ascomycetes, the aspergilli form septate mycelia. During sexual reproduction a fusion of certain cells results in the formation of hollow, flasklike or gourdlike bodies called *perithecia* which contain asci with spores (Fig. 93). It is for this reason that the aspergilli are included in the same order (Ascomycetes) as the yeasts.

**Perithecium Formation.**—In the formation of a perithecium two hyphal cells twist about each other. The separating walls dissolve and the resulting fusion-cell gives rise to a nodule of branched hyphae among which a mass of asci or spore sacs is formed. The whole mass is often enclosed in a sort of hull or protective cellular covering formed by the surrounding mycelium. Each ascus, as a rule, contains eight ascospores.

**The asexual fruiting bodies**, or conidiophores, of aspergilli consist of tall hyphae which have an enlarged globular tip. From the surface of this project numerous small stems called *sterigmata*, and on the tips of these the conidia are borne in long chains (Fig. 94, D). There is no sac or envelope as in the mucors. Classifications of aspergilli are based to some extent on the structure, but more particularly on the color, of the conidial heads.

**Common Species.**—One of the commonest species of *Aspergillus* is *A. glaucus*. It forms green or grey-green conidia and yellow perithecia and is frequently seen on bread, preserves and clothing during the summer or in the tropics. Another common environmental species, sometimes found as a laboratory nuisance, *A. nidulans*, may be confused with *Trichoderma koningi* because of its bright green color. The perithecia are reddish in color. *A. niger*, also an extremely common species, is recognizable by its very large round masses of black conidia. It is sometimes confused with *Rhizopus nigricans* which also produces black sporangia.

There are some pathogenic species. For example, a lung infection of birds due to *Aspergillus fumigatus* is not uncommon. However, the molds of the genus *Aspergillus* are chiefly of importance as scavengers and are found, usually along with other molds and

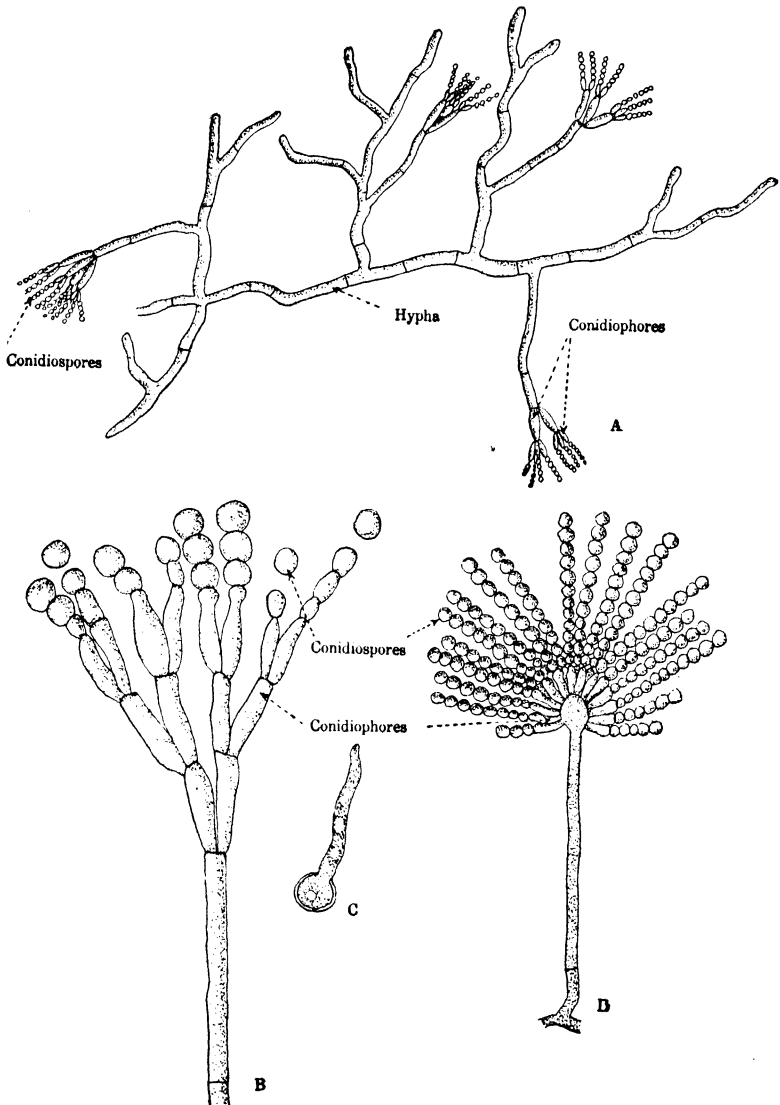


Fig. 94.—Blue and green molds. *A* and *B*, Common blue mold (*Penicillium*); *C*, germinating conidiospore; *D*, green mold (*Aspergillus*). (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc., publishers.)

bacteria, busily engaged in the decomposition of a great variety of waste matter. On the other hand, due to this ability to decompose organic matter these organisms sometimes cause great economic loss through the spoilage of foods, wood, paper, and other commodities composed of organic matter. The well-known "moldy" odor characteristic of some damp cellars is due in part to the growth and metabolic products of these organisms.

**Genus *Penicillium*.**—The penicillia are very common in Nature and contribute to the spoilage of various objects and materials composed of organic matter, especially ripe fruits. Like the aspergilli, the mycelia are septate in structure.

The conidiophores are composed of hyphae which branch extensively at the tip into clusters called *verticils*, the whole roughly suggestive of the bony structure of the hand. The spores extend in parallel chains from the ends of the "fingers" (*sterigmata*). This arrangement gives the whole conidiophore a form suggestive of a tiny paint brush, from which the generic name is derived (Fig. 94, *A* and *B*).<sup>8</sup> As in other groups of molds, the color and form of the fruiting body furnish characters of differential value in classification. Sacs or perithecia are formed by penicillia in much the same manner as by the aspergilli, but some species of *Penicillium* are classed as *Fungi imperfecti*.

Some species of *Penicillium* are differentiated chiefly by their habitat. Thus, the green molds found in Roquefort cheese (*P. roqueforti*), Camembert cheese (*P. camemberti*) and other cheeses of the same nature are distinguished chiefly because of their occurrence there. The molds grow in or on the cheese, producing various enzymatic changes in the fat, carbohydrate and protein of the cheese which result in characteristic aromas, flavors and textures. *P. roqueforti* grows well under conditions of temperature and humidity found in the limestone caverns in the province of Roquefort, France. It is micro-aerophilic, growing in the interior of the cheese masses provided holes are punched in the cheese to admit small amounts of air. (These holes must not be confused with the holes in Swiss cheese produced by the action of the *Propionibacter*.)

*Penicillium camemberti*, which closely resembles *P. roqueforti*, is strictly aerobic and grows only on the exterior of Camembert cheese, so that the ripening of this delicacy proceeds from without inward (see page 579).

There are many other species of penicillia and they are frequently seen on old bread, cheese, lemons, and other fruits. They may usually be recognized as members of the *genus* by their sky-blue

or green color. The individual *species* may be differentiated by the various arrangements of the conidiophores.

*Penicillium notatum*.—This species, a not uncommon laboratory contaminant, very much like *P. camemberti*, etc., has recently come into great prominence as a source of what promises to be one of the best drugs for use in infections of the blood and tissues.<sup>8</sup> The active principle, called *penicillin*, is given off as a waste product into ordinary medium during growth. It is more fully discussed in the section on disinfection (see page 132).

**Fungi imperfecti.**—The Fungi imperfecti constitute a group of which the reproductive cycle is not completely known. The membership of the group is, therefore, rather heterogeneous since imperfect forms of Ascomycetes, Phycomycetes, Basidiomycetes, etc., are numerous. The largest number of species of Fungi imperfecti, however, are really Ascomycetes of which our knowledge is incomplete.

Classification of Fungi imperfecti must be based on asexual structures and for this reason genera of Fungi imperfecti are not necessarily composed of related species. Inasmuch as there are about 1,330 genera and some 20,000 species, a complete discussion of the Fungi imperfecti is out of the question. We may, however, give attention to a few common species of filamentous fungi of this group which any bacteriologist may from time to time encounter in his laboratory work.

- I. *Sporotrichum*. Conidia oval or saclike, occurring singly or in clusters at all parts of the mycelium; attached by pedicles or stems; never in chains; never on conidiophores.
- II. *Trichoderma*. Conidia occur in compact masses on tips of well-developed conidiophores which branch like trees or shrubs; bright green in color; common in soil and as contaminant in laboratory.
- III. *Oidium* and *Monilia*. Reproduce chiefly by fragmentation of the filaments into yeastlike cells (oidia), vegetative cells, or by the formation of conidia. Have both yeastlike and moldlike characters.
- IV. *Aspergillus*.<sup>\*</sup> Round or oval conidia produced in unbranched chains from sterigmata which radiate from a central, unbranched, club-shaped conidiophore, new conidia formed at base of chain.
- V. *Penicillium*.<sup>\*</sup> Round or oval conidia produced in unbranched

<sup>\*</sup> Most of the *Aspergillus* and *Penicillium* species are Fungi imperfecti. As these genera have already been described they are not discussed here.

chains from clustered, digital branches of the conidiophore; new conidia formed at *base* of chain.

- VI. *Hormodendron*. Conidiophore produces one conidium, which reproduces *apically*, branching as the chains of conidia form.
- VII. *Alternaria*. Conidia contain 8 to 12 or more large cells in a conical group, the divisions between the cells being in various planes; the groups occurring in chains, or spaced along the mycelium.

**Genus *Sporotrichum*.**—There are several saprophytic species of *Sporotrichum*, among which *S. schenckii* is of particular interest because, in addition to living a saprophytic existence, attached to grasses and other plants, it also causes a disease of man known as sporotrichosis.

In the latter circumstance it probably gains entrance to the skin or mucous membranes and lymphatic spaces by way of wounds made by straw, thorns and similar objects. It grows along the lymph spaces, producing rows of red nodules which sometimes



Fig. 95.—Sporotrichosis. (Reprinted by permission, from Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., publishers.)

ulcerate (Fig. 95). There is little pain or soreness. Sporotrichosis lesions superficially resemble those of tertiary syphilis and special care should be taken in areas where sporotrichosis occurs, to make certain of the diagnosis. In the pus, when stained by Gram's method, the organisms are seen in the pus cells as elliptical, gram-positive bodies about the size of some bacteria of the genus *Bacillus* ( $2\ \mu \times 8$  or  $10\ \mu$ ). The disease has been described in horses in the Army.<sup>9</sup>

*Sporotrichum* may be isolated from such material on one of the media adapted for the growth of molds, preferably Sabouraud's agar. The colonies resemble those of bacteria, but contain mycelial filaments closely packed. They turn brown with age. Aerial mycelia are seldom formed. The forms of conidia and hyphae are seen in Figure 96.

**Genus Trichoderma.**—The best-known species of this genus is *T. koningi*, a common soil and environmental species which often enters laboratory cultures uninvited. It is of a bright green color with well-developed aerial mycelium (Fig. 97). It forms large amounts of ammonia, and an ammoniacal odor gives a clue to its identity.

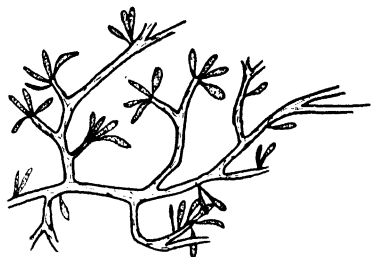


Fig. 96.—*Sporotrichum schenckii*.

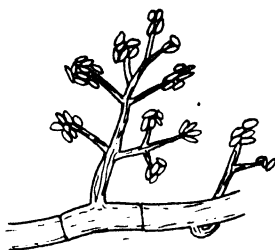


Fig. 97.—*Trichoderma koningi*. (Redrawn from Henrici.)

**Genera Oidium and Monilia.**—As indicated above, the organisms of these genera are moldlike in that at times they form mycelial growths while at other times they form yeastlike cells (Figs. 86 and 98), the two sorts of cell sometimes occurring simultaneously in

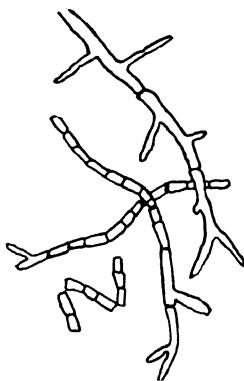


Fig. 98.—*Oidium lactis*. Note various forms of cells

the same culture or pathological lesion. Due to confusion arising from this instability of form, the generic terms *Oidium* and *Monilia* are used almost as though they were synonymous by many writers, and authorities are quite at variance as to the exact systematic

position of organisms of this type. According to Henrici and others, the term *Oidium* should apply only to those species which form free, yeastlike cells (oidia) by *fragmentation* of the mycelium; while the term *Monilia* would include the species in which free, yeastlike cells are formed from the mycelium only by a process of actual *budding*. If these characters remain *fixed* and *exclusive* in the various species to be classified, the system should be helpful.

Many species of *Monilia* and *Oidium* are known, some of them being common contaminants in the laboratory, but only three will



Fig. 99.—Stained preparation of pus from a case of generalized blastomycosis, showing two of the double-contoured cells of *O. dermatitidis*. (Ninho.)

be mentioned here. One, representing the harmless types, is *Oidium lactis* (Fig. 98).

*Oidium lactis*.—This very readily metabolizes lactic acid and is of common occurrence in soured dairy products, like sour milk, cheese or butter, and in sauerkraut and silage where lactic acid is formed by other organisms in these products. Like most other molds, it is metabolically versatile, attacking proteins and many other compounds, producing ammonia and causing spoilage in many fermented products. It grows as a white, felt-like, adherent membrane on the surface of solid media like Camembert and other cheeses, clotted milk or agar. The aerial mycelium which forms as

the plant grows older, and which is distinguished by *dichotomous* branching, is often quite distinct and conidia are borne in long chains. Lactic acid is necessary for any extensive growth.

*Monilia albicans* (also called *Oidium albicans*) is a well-known species commonly associated with, and probably the cause of, thrush, a disease of the mucous membranes of the mouth and gastro-intestinal tract and sometimes of the vagina. There are local inflammation and white, membranous patches. Microscopic examination of scrapings from the affected parts reveals the fila-

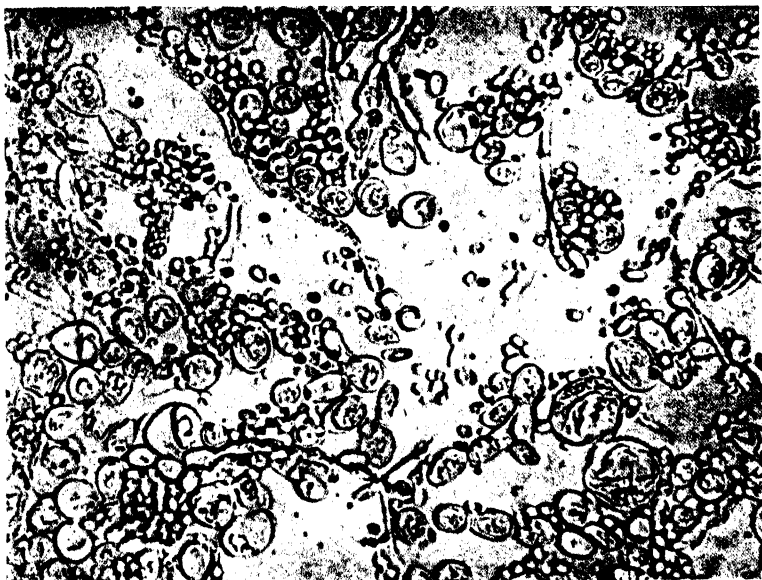


Fig. 100.—Unstained, moist preparation of yeast (probably *O. dermatitidis*) cultivated from a case of blastomycosis.

ments and yeastlike cells. A somewhat similar disease of the gastro-intestinal tract, sprue, is thought by some to be due to *Monilia albicans* or a species very much like it, *M. psilosis*. However, these organisms occur very widely in normal individuals, and their relations to sprue and thrush are questioned by many.

*Oidium dermatitidis*, also widely known as *Cryptococcus gilchristi* and *Blastomyces dermatitidis*, is a dangerous pathogen, causing the disease known as blastomycosis (Fig. 87). Because of the appearance of large, thick-walled, yeastlike cells (Fig. 99), readily seen



in pus treated with 20 percent potassium hydroxide and examined wet under cover slips, the organism was originally regarded as a true yeast. The fact that the cells reproduce by budding supported this idea. Later observations of the development, on artificial media, of filamentous growth showed that it belongs to the group of oidia (Fig. 100).

*Coccidioides immitis*, a superficially similar species, is the cause of a disease called coccidioidomycosis occurring chiefly in the San Joaquin Valley of California. As in the history of many diseases, only the severe and fatal (relatively rare) cases were recognized at first, especially the clinical form known as coccidioidal granuloma, a condition in some respects resembling blastomycosis and in some tuberculosis. In recent years, however, it has been found by Charles E. Smith,<sup>5a</sup> Aronson,<sup>4</sup> and others that a large number of infections occur which result in a mild febrile disease in association with bronchitis or pneumonia. Many patients develop a nodular eruption, called erythema nodosum, which appears to be an allergic reaction, during the progress of the disease. The mild febrile form of the disease has for long been known in the San Joaquin Valley as "valley fever." In many respects the relatively mild respiratory form of the disease resembles tuberculosis and may often have been confused with it.

A useful means of diagnosis consists in the coccidioidin test, which is based upon the same principle as the tuberculin test, *i.e.*, it is an allergic reaction. That the infection may be more widespread than was formerly supposed was shown in 1941 and 1942. In an examination by means of the coccidioidin test it was found that 2.5 percent of 680 students in a school in an eastern state gave a positive reaction to coccidioidin. Eight had never been west of the Mississippi river.<sup>4,4a</sup> An outbreak of the disease among troops on maneuvers was reported in 1943.<sup>5</sup>

The causative organism resembles *Oidium dermatitidis* in some morphological details and in metabolism. However, it is readily differentiated by cultural and microscopic examination, as shown by Stewart and Meyer.<sup>4a</sup>

When *C. immitis* is cultivated in the absence of free oxygen, or when invading the tissues of the body, it forms cells resembling those of *O. dermatitidis*, but they never form buds. On the contrary, the cell contents divide into many smaller cells somewhat suggestive of very exaggerated ascus formation, and the mother-cell wall ruptures, liberating large numbers of the small cells which are then scattered by the blood throughout the body and which repeat the

cycle. When cultivated aerobically, mycelial filaments are formed which resemble oidia. No conidia are formed (Fig. 101).

When growing in the mycelial form, some of the oidia acquire thick protective walls and apparently go into a drought- and sunlight-resistant resting form, like a chlamydospore. It is in this form that the organism is blown about in dry dust, apparently growing vegetatively as a saprophyte in the soil on decomposing organic matter. There is evidence that infection occurs only through the agency of the chlamydospores. The disease is especially prevalent in regions and seasons marked by the presence of much

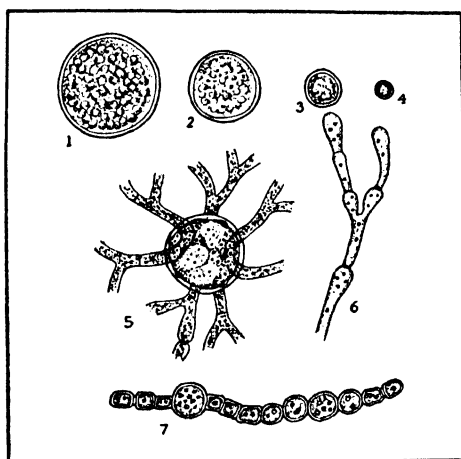


Fig. 101.—*Coccidioides immitis* ( $\times 250$ ). 1 to 4, Cells containing ascospores in tissues; 5, development of mycelium from large round cell; 6, mycelium in culture; 7, old mycelium with chlamydospore. (After MacNeal, Taylor and Moore, from Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

alkali dust in the air, such as occurs in the San Joaquin valley and elsewhere during harvest time.<sup>5a</sup>

**Genus *Alternaria*.**—Several kinds of dark green or brownish-green molds are often met with as contaminants in laboratory media and on Petri dish cultures. Among the commonest forms are members of the genus *Alternaria*. They are not so large and extensive as *Aspergillus* or *Penicillium*. The colonies of *Alternaria* are more compact, and the underside and mycelium are very dark colored. The conidia are relatively large, and occur in roughly conical or ellipsoidal masses in which they are packed together so

that they are irregularly shaped. These groups of conidia are often arranged in long rows, or occur along the mycelial filaments (Fig. 102).

**Genus *Hormodendron*.**—These molds, like *Alternaria*, form relatively small, dark green or brownish-green colonies with a fine,

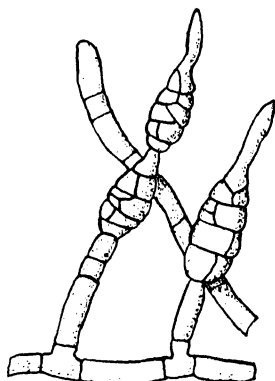


Fig. 102.—*Alternaria*, showing muriform multicellular conidia.

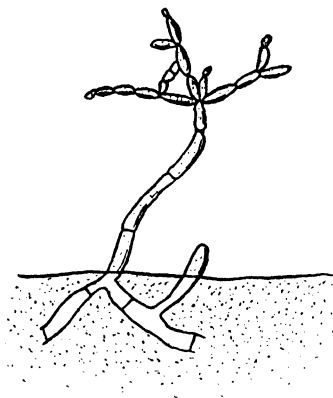


Fig. 103.—*Hormodendron*; dark, smoky or black colonies.

close, velvety surface. They produce conidia by growth at the *tip* of the chains (apically) rather than at the base of the chains as in *Aspergillus* and *Penicillium*. Thus, in contrast to the last two, the youngest conidia in *Hormodendron* are at the tips. The conidia are rather oval or elongated, and the chains of conidia are often branched.

## THE DERMATOPHYTES

Occasionally certain species of *Aspergillus* and *Penicillium* invade tissues, especially of the lungs of the human and animal body, producing what are called mycotic infections or mycosis. Such infections are serious and often fatal, but are fortunately rather rare. Mycotic infections of the skin are far more common; in fact skin mycoses are among the commonest of infectious diseases. The molds involved are a rather well demarked group called *dermatophytes*. In general they belong in the Fungi imperfecti but there are some exceptions. A convenient classification of dermatophytes would include

## I. Yeastlike forms.

*Torula* (meningitis; European blastomycosis)

*Oidium* (Thrush; moniliasis; paronychia; vaginitis) (*Monilia*)

## II. Filamentous forms.

## A. Ringworm (tinea) fungi.

1. *Microsporum*

*M. canis* (animal type: tinea capitis in children)

*M. audouini* (human type: tinea capitis in children)

*M. gypseum* (tinea)

2. *Trichophyton* (Achorion)

*T. schoenleini* (Favus)

*T. gypseum* (Kerion; tinea sycosis)

*T. rubrum*; *T. rosaceum*; *T. violaceum*

(various forms of tinea, kerion, etc).

*T. tonsurans* ("barbers itch" or tinea capitis)

3. *Epidermophyton*

*E. floccosum* (mycosis of hands, feet and groins. Similar conditions caused by various *Trichophyton* species).

## B. Other pathogenic filamentous fungi.

- |                             |                            |
|-----------------------------|----------------------------|
| 1. <i>Coccoides immitis</i> | } Previously<br>discussed. |
| 2. <i>Sporotrichum</i>      |                            |

For a complete discussion of fungus infections the student should consult a textbook on Medical Mycology.<sup>6, 7</sup> Here we may mention two of the commonest mycoses, *tinea* or ringworm, and various mycotic infections of the hands and feet, the last including the various conditions collectively known as "athlete's foot."

In examining suspected material from tinea capitis (scalp ringworm) stumps of hairs and contents of abscesses are observed. In tinea of the smooth skin, or in mycosis of the hands and feet, scrapings of the affected skin are taken around the margins or from the inside of the covering of vesicles. The material is placed on a slide in a drop of 25 percent potassium hydroxide, covered with a

cover slip and warmed gently for a minute. It may be examined after about 20 minutes. Permanent preparations may be made by washing and staining the material. Cultures may be made on Sabouraud's medium or one of many modifications of it. A very useful test for the identity of species of *Microsporum* is to mingle the mycelia in cultures. Mycelium of *M. canis* will fuse with mycelium of this species, but not with those of *M. audouini*. Other tests for the identity of various dermatophytes include fermentation tests and examination by ultraviolet light. In a dark room ultra-

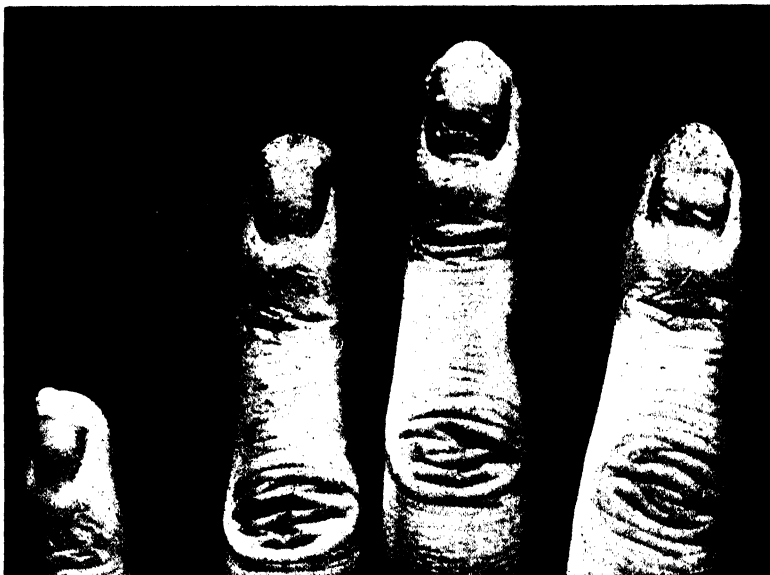


Fig. 104.—Chronic paronychia caused by *Monilia albicans*. (From Swartz & Reilly, "Diagnosis and Treatment of Skin Diseases," by permission of The Macmillan Company.)

violet rays directed on suspicious patches on scalp or elsewhere will fluoresce due to the fluorescent properties of the fungi in the lesion. Various structures seen in dermatophytes are shown in Figure 107. They are well described also by Emmons.<sup>10</sup>

*Tinea*.—Ringworm may be caused by one of several species of fungi as outlined above. It is common in children. *Tinea capitis* produces loss of hair, with broken hairs, "black heads" and dandruff. *Tinea circinata* is found on the smooth skin of face, neck and arms and is a tropical "ringworm." *Tinea sycosis* involves the

beard. There are many forms of tinea, all due to closely related fungi.

"*Athlete's Foot*."—This is a term covering various mycotic infections when the lesions are especially manifest in the feet. Infection may occur as a result of walking barefoot in crowded gymnasias,



Fig. 105.—Moniliasis of foot. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

bathing houses, etc., where the floors are warm and wet and the organisms can survive and be readily transferred from one person to another. Various causative agents of the *Microsporum* and *Trichophyton* groups are involved. Cleanliness of feet, and care in avoiding infected places with bare feet, will aid in preventing

mycotic infections of the feet. As the same organisms also invade the skin of the crotch or groin and adjacent areas, care in using clean, disinfected clothing, suspensories, and towels is desirable in

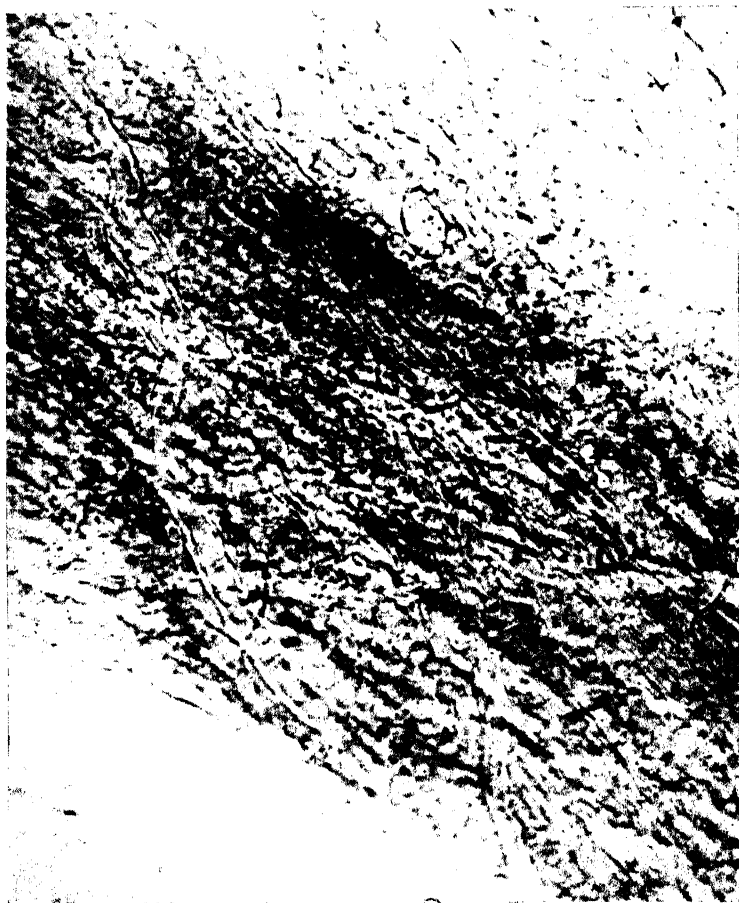


Fig. 106.—Spores and mycelium in potassium hydroxide preparation of hair in tinea capitis. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

all persons, especially athletes. Persons who perspire much are more likely to contract mycotic dermatitis than are others.

Some of the lesions due to various dermatophytes, and some of the causative organisms, are seen in Figures 104 through 111.

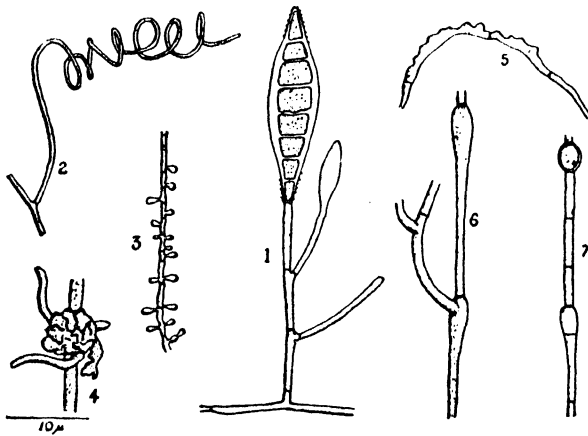


Fig. 107.—1. Mature macroconidium (fuseau) seen in optical section (immature macroconidium begins as swelling at apex of side branch). 2. Spiral hyphae. 3. Laterally borne microconidia. 4. Nodular body. 5. Pectinate hypha. 6. Racquet hypha. 7. Chlamydospore, thick-walled resting spore. Spirals exceptionally rare. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

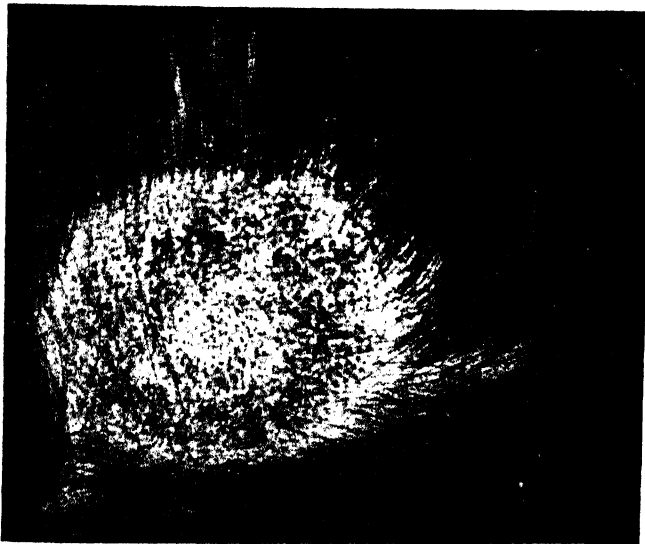


Fig. 108.—Tinea capitis caused by *Trichophyton*. (From Swartz and Reilly, "Diagnosis and Treatment of Skin Diseases" by permission of The Macmillan Company.)





Fig. 109.—*Tinea circinata*. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

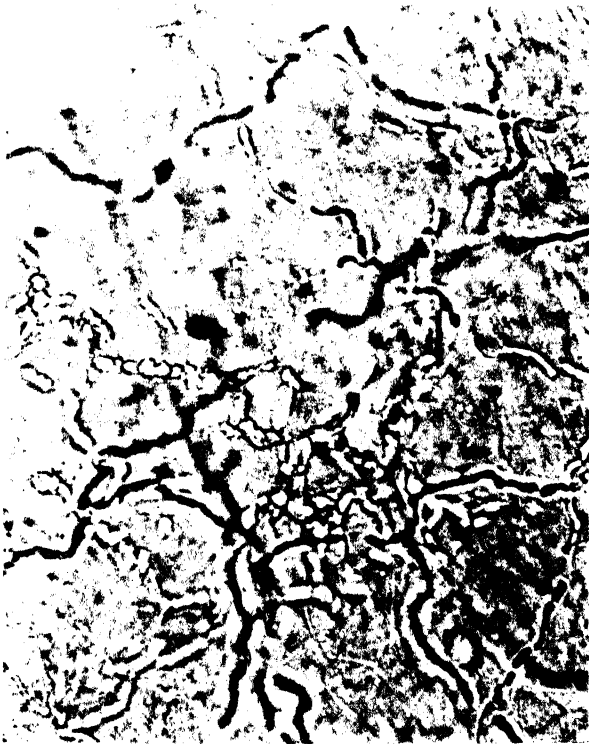


Fig. 110.—Mycelia and mosaic structures in scales. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)



Fig. 111.—Dermatophytosis of foot. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

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## CHAPTER 10

### CLASSIFICATION OF BACTERIA

ONE of the aims of science has always been to organize and systematize knowledge and phenomena so that their true relations and significance can be clearly seen. Objects may be arranged in order of importance, size, shape, or some other criterion. In dealing with small numbers of objects having clearly defined characters on which to base the arrangement, this is not too difficult a task. In the example given above, however, the concept of *importance* is used. Here arises a very grave difficulty. Whose opinion as to grades of importance is to be accepted? It is obvious that, if many facts or objects of poorly differentiated or nearly equal importance are involved, and more than one evaluation of relative degrees of importance are made, the orderly arrangement becomes a matter of great complexity and may never be settled to the satisfaction of all.

In bacteriology, as in other branches of biology, the problem of classification is complicated not only by a multiplicity of characteristics of different bacteria on which to base a classification, but wide disagreement as to which characteristics are of most importance. Even if the important characters were finally agreed upon, a new difficulty would arise because many of them are variable and an organism having one set of characters today may have others tomorrow. This would be a serious obstacle by itself, but it is still further complicated by the fact that some species, ordinarily rather similar, in their variant phases may assume some of the characters of others so that the taxonomist (one who attempts to bring order out of such seeming chaos) finally sets up arbitrary divisions among the creatures he is classifying and rules that the possession of certain characters shall differentiate between such and such species. This is convenient until it is found that certain of the creatures have lost some of the characters given, but have acquired others. A further element of uncertainty is found in inaccurate descriptions of some organisms so that they get put into the wrong group or seem to fit into no group. Probably no universally acceptable classification of bacteria, or of any other living forms for that matter, will be constructed during our lifetime.

As bacteriologists continue their studies new species are found, more and more information comes to light regarding the various organisms, and it becomes necessary, from time to time, to sub-

divide and rearrange groups and give a new name to each of the subdivisions. Thus the systems of classification are enlarged and names and groupings corrected in light of new knowledge each year. The selection of the proper name for an organism requires a knowledge of the rules of nomenclature, of previously used names, and an exhaustive knowledge of the characteristics of the organism and of similar organisms already named.

*Species and Genera.*—In bacteriology the terms species and genus are used but the concept of these is somewhat vague since we have no knowledge of genetics in relation to bacteria. In bacteriology, a species is theoretically a single kind of bacterium all individual cells of which are identical or nearly so. In actuality this identity of cells rarely exists. In any culture of a given species cells may be found which, while having the outward form, staining properties and other characters of most of the cells in the group, possess different metabolic properties, different antigenic composition and so on. Usually these differences are not extreme and may represent only temporary fluctuations from the principal type. When two bacteria have one or more *well-marked* morphological, metabolic or other differences between them which are *constant*, the two may be regarded as distinct species. But the same differences may be used as a basis of generic or tribal or even familial distinction between some other kinds of bacteria. The species concept in bacteriology, it must be admitted, is built on somewhat vague lines.

The concept of genera is, in many instances, equally nebulous. A genus is theoretically and ideally a group of species all of which bear sufficient resemblance to one another to be considered closely related and easily distinguishable from members of other groups or genera. The boundaries of some genera are sharply defined by as few as three characteristics, as in the genus *Bacillus*: (1) aerobic; (2) spore-forming; (3) rods. The boundaries of other genera are sometimes more difficult of definition; for example, the genera *Salmonella*, *Escherichia*, *Shigella*, and *Aerobacter*, all of which are non-sporeforming, gram-negative, facultative, aerobic rods. An organism of one genus may thus possess several of the important (?) characters of two or three or more other genera and its proper allocation to one of these is often difficult and must be decided on a more or less arbitrary basis.

**Bacterial Nomenclature.**—In naming a bacterium, certain definite rules are followed. Each species is allowed a "first" and "last" name only. This two-name scheme is called the binomial system

and originated in 1760 under the leadership of Linnaeus. The first name of a bacterium refers to the genus, and is usually a Latin or latinized word (sometimes Greek), generally a noun, based on the morphology of the organism or on the name of the discoverer. It is written with a capital letter. The last name is the species name and is usually descriptive of the noun, referring to its color, source, disease production, discoverer or some other distinguishing point. It is not capitalized. Genus and species names are generally italicized. For example, the name *Bacillus anthracis* indicates that the organism is a spore-bearing, aerobic rod (properties of the genus *Bacillus*), while *anthracis* calls attention to the fact that this species of the genus *Bacillus* produces the disease anthrax. The name *Spirillum rubrum* shows that the organism is a true saprophytic bacterium, rigid, spiral in structure, non-sporeforming, motile, and gram-positive (all properties of the genus *Spirillum*), and that the species named is characterized by a red color (*rubrum*). The name *Clostridium novyi* indicates a gram-positive, spore-forming, rod-shaped organism, saprophytic or parasitic, and restricted to growth in the total absence of *free oxygen*. These are properties of the genus *Clostridium*. This particular species bears the name *novyi* in honor of Dr. F. G. Novy of the University of Michigan, who discovered the organism and its relation to a disease, gas gangrene. The practice of using personal names for newly discovered species of bacteria is obsolescent, although many generic names are derived from the discoverers or original students of the genus, for example the genus *Salmonella*, from a famous American bacteriologist named Salmon.

In writing of bacteria, it is customary to abbreviate the generic name, using only the initial letter if it is clear what genus is meant, for example *B. anthracis* for *Bacillus anthracis*. Sometimes the abbreviation may be longer, as *Br. abortus* for *Brucella abortus*. Medical bacteriologists often dispense with these rules and designate organisms by the disease with which they are most frequently associated, for example, meningococcus, pneumococcus, typhoid bacillus, etc. This is a convenient but loose custom not in accord with rules of nomenclature.

**Systems of Classification.**—Shortly after Leeuwenhoek's discovery of bacteria, scientists began to classify the living things they saw with microscopes. The bases of their classifications were size and shape (morphology) because they had little or no other information to use. Thus they confused bacteria, protozoa, algae, etc. Muller, in 1786, listed two groups, *Vibrio* and *Monas*, which

probably were bacteria. For many years morphology was the principal basis on which the large subdivisions of microscopic creatures were founded. Color, an easily determinable character, was also used as a basis for subdivisions within groups. Arrangement of cells, as in chains, pairs or clumps, was another. Motility, readily visible with the microscope, was still another, and habitat another. Thus many of the older names in bacterial nomenclature are based on readily *visible* characteristics and on habitat.

**Biochemistry and Taxonomy.**—With the increase in knowledge of chemistry in general, there came an increase in knowledge of the biochemistry of bacteria. When Koch showed the way to pure culture study, it became possible to differentiate morphologically identical organisms by their biochemical properties. These properties soon found their way into taxonomy as the bases of more accurate subdivisions of previously large and heterogeneous groups and today many generic and species distinctions are based on biochemical properties of morphologically indistinguishable organisms.

**Antigenic Structure and Taxonomy.**—In attempts to introduce still greater accuracy into systems of classification, immunology has been drawn upon, and minute antigenic differences between organisms believed to constitute a single species are now sometimes used to formulate new species. Often, however, serological subdivisions of a species are designated as *types*, like pneumococcus types I, II, III, etc., or *groups*, as Lancefield group A, B, C, etc., of streptococci.

**Classification Schemes.**—Many systems of classification have been brought forward during the last two centuries, but none has remained long without revision and enlargement. A system widely used by American bacteriologists and which has international standing is published under the editorial direction of Breed, Murray and Hitchens in a volume generally known as "Bergey's Manual." The book represents the collaborative effort of many of the world's best bacteriologists. This system, in 1939, divided the entire group of bacteria (Class Schizomycetes) into 7 major subdivisions or orders, which are outlined below. Each order is divided into families, and these into tribes, genera and species. Groups of similar species constitute genera; groups of similar genera constitute tribes, and so on. The names of orders end with the suffix *ales*; names of families with *aceae*; names of tribes end with *eae*.

Some species and genus names change with revision of the systems of classification, and different names are therefore some-

times used by different authors for the same organism, or two different organisms may be called by the same name. Thus there is some confusion in bacterial literature. This is one of the signs of progress.

## ORGANISMS OF THE CLASS SCHIZOMYCETES<sup>2</sup>

Kingdom: *Vegetable*

Subkingdom: *Thallophyta*

Division: *Eumycetes*

Class: **Schizomycetes**

### ORDER I. EUBACTERIALES

Family I. *Nitrobacteriaceae*

Tribe I. *Nitrobacteriaceae*

Genus I. *Nitrobacter*

Genus II. *Nitrosomonas*

Genus III. *Nitrosococcus*

Tribe II. *Protobacteriaceae*

Genus IV. *Hydrogenomonas*

Genus V. *Methanomonas*

Genus VI. *Carboxydomonas*

Tribe III. *Thiobacillaceae*

Genus VII. *Thiobacillus*

Family II. *Rhizobiaceae*

Genus I. *Rhizobium*

Genus II. *Chromobacterium*

Genus III. *Alcaligenes*

Family III. *Pseudomonadaceae*

Tribe I. *Spirillaceae*

Genus I. *Vibrio*

Genus II. *Cellvibrio*

Genus III. *Cellfalcicula*

Genus IV. *Spirillum*

Tribe II. *Pseudomonadeae*

Genus V. *Pseudomonas*

Genus VI. *Phytomonas*

Genus VII. *Protaminobacter*

Genus VIII. *Mycoplasma*

Family IV. *Acetobacteriaceae*

Genus I. *Acetobacter*

Family V. *Azotobacteriaceae*

Genus I. *Azotobacter*

Family VI. *Micrococcaceae*

Genus I. *Micrococcus*

Genus II. *Staphylococcus*

Genus III. *Gaffkya*

Genus IV. *Sarcina*

Family VII. *Neisseriaceae*

Genus I. *Neisseria*

Genus II. *Veillonella*



**Family VIII. *Parvobacteriaceae*****Tribe I. *Pasteurellae***Genus I. *Pasteurella*Genus II. *Malleomyces***Tribe II. *Brucellae***Genus III. *Brucella***Tribe III. *Hemophilae***Genus IV. *Hemophilus*Genus V. *Noguchia*Genus VI. *Dialister***Family IX. *Lactobacteriaceae*****Tribe I. *Streptococcaceae***Genus I. *Diplococcus*Genus II. *Streptococcus*Genus III. *Leuconostoc***Tribe II. *Lactobacillae***Genus IV. *Lactobacillus*

Subgenera

*Thermobacterium**Streptobacterium**Betabacterium*Genus V. *Propionibacterium***Family X. *Enterobacteriaceae*****Tribe I. *Eschericheae***Genus I. *Escherichia*Genus II. *Aerobacter*Genus III. *Klebsiella***Tribe II. *Erwineae***Genus IV. *Erwinia***Tribe III. *Serrateae***Genus V. *Serratia***Tribe IV. *Proteae***Genus VI. *Proteus***Tribe V. *Salmonellae***Genus VII. *Salmonella*Genus VIII. *Eberthella*Genus IX. *Shigella***Family XI. *Bacteriaceae***Genus I. *Listerella*Genus II. *Microbacterium*Genus III. *Kurthia*Genus IV. *Cellulomonas*Genus V. *Achromobacter*Genus VI. *Flavobacterium*Genus VII. *Actinobacillus*Genus VIII. *Bacteroides*Genus IX. *Fusobacterium*Genus X. *Bacterium***Family XII. *Bacillaceae***Genus I. *Bacillus*Genus II. *Clostridium*

## ORDER II. ACTINOMYCETALES

Family I. *Mycobacteriaceae*Genus I. *Corynebacterium*Genus II. *Mycobacterium*Family II. *Actinomycetaceae*Genus I. *Leptotrichia*Genus II. *Erysipelothrix*Genus III. *Proactinomyces*Genus IV. *Actinomyces*

## ORDER III. CHLAMYDOBACTERIALES

Family I. *Chlamydobacteriaceae*Genus I. *Sphaerotilus*Genus II. *Clonothrix*Genus III. *Leptothrix*Genus IV. *Crenothrix*

## ORDER IV. CAULOBACTERIALES

Family I. *Nevskiaceae*Genus I. *Nevskia*Family II. *Gallionellaceae*Genus I. *Gallionella*Family III. *Caulobacteriaceae*Genus I. *Caulobacter*Family IV. *Pasteuriaceae*Genus I. *Pasteuria*Genus II. *Blastocaulis*

## ORDER V. THIOBACTERIALES

Family I. *Rhodobacteriaceae*Subfamily I. *Chromatioideae*Tribe I. *Thiocapsee*Genus I. *Thiocystis*Genus II. *Thiosphaera*Genus III. *Thiosphaerion*Genus IV. *Thiocapsa*Genus V. *Thiosarcina*Tribe II. *Lamprocysteae*Genus I. *Lamprocystis*Tribe III. *Thiopédieae*Genus I. *Thiopedia*Genus II. *Thioderma*Genus III. *Lampropedia*Tribe IV. *Amoebobacterieae*Genus I. *Amoebobacter*Genus II. *Thiodictyon*Genus III. *Thiothece*Genus IV. *Thioplyococcus*Tribe V. *Chromatieae*Genus I. *Chromatium*Genus II. *Rhabdomonas*Genus III. *Thiospirillum*Genus IV. *Rhodocapsa*Genus V. *Rhodotheca*

Subfamily II. *Rhodobacterioideae*

- Genus I. *Rhodocystis*
- Genus II. *Rhodomonas*
- Genus III. *Rhodorhagus*
- Genus IV. *Rhodobacterium*
- Genus V. *Rhodobacillus*
- Genus VI. *Rhodovibrio*
- Genus VII. *Rhodospirillum*

Family II. *Beggiatoaceae*

- Genus I. *Thiothrix*
- Genus II. *Beggiatoa*
- Genus III. *Thioploca*

Family III. *Achromatiaceae*

- Genus I. *Achromatium*
- Genus II. *Thiophysa*
- Genus III. *Thiospira*
- Genus IV. *Hillhousia*

## ORDER VI. MYXOBACTERIALES

Family I. *Archangiaceae*

- Genus I. *Archangium*
- Genus II. *Stelangium*

Family II. *Sorangiaceae*

- Genus I. *Sorangium*

Family III. *Polyangiaceae*

- Genus I. *Polyangium*
- Genus II. *Synangium*
- Genus III. *Melittangium*
- Genus IV. *Podangium*
- Genus V. *Chondromyces*

Family IV. *Myxococcaceae*

- Genus I. *Myxococcus*
- Genus II. *Chondrococcus*
- Genus III. *Angiococcus*

## ORDER VII. SPIROCHAETALES

Family I. *Spirochaetaceae*

- Genus I. *Spirochaeta*
- Genus II. *Saprosira*
- Genus III. *Cristispira*
- Genus IV. *Borrelia*
- Genus V. *Treponema*
- Genus VI. *Leptospira*

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## CHAPTER 11

### METHODS OF SYSTEMATIC STUDY

**Variation and Identity.**—In view of the variability of bacteria, mentioned in the preceding chapter, it might be thought that the exact identification and description of species of bacteria would be a useless if not impossible task. This is not the case because variation usually occurs within certain fairly well-known limitations. Practically all forms of life vary to some extent, some more than others. Creatures with which we are thoroughly familiar may vary considerably yet we recognize them without difficulty because (a) we are familiar with the variant forms, and (b) we recognize certain basic similarities among the variants. We might show a giant, 8-inch, single, plain, yellow zinnia growing 5 feet tall and a dwarf,  $\frac{1}{2}$ -inch, double pompom zinnia only 6 inches tall with striped, curled petals to a person who had never seen or heard of a zinnia of any kind and he might doubt their membership in the same genus. But the average flower grower, to whom such unlikenesses are commonplace, recognizes similarities of leaf, stem, root, odor, and a dozen other features which leave him in no doubt as to the identity and relationship of the two plants. Further, he knows that zinnias, however much they vary as to size, shape and color, always retain the basic properties of zinnias and do not change into roses or ragweed.

So with bacteria; although they may at times vary greatly in one or more respects, they seldom do so in all details at once and, normally, under the uniform conditions of laboratory study, they remain quite recognizable as bacteria, and retain fundamental, species-distinguishing characteristics. It is necessary to know what these are, and how they are usually determined in the laboratory.

From descriptions given earlier in this book there should be no difficulty in identifying an unknown organism as a bacterium and determining that it is not a protozoan, a yeast, a mold or an alga. Having proceeded so far, the next step is discretionary, depending on information available about our organism, and what we wish to learn concerning it.

**Determination of Nutrient and Temperature Requirements.**—Nearly always some information as to source of the unknown is available and greatly narrows the possibilities. For example, we would not attempt autotrophic cultivation of a bacterium obtained

from the spinal fluid in a case of meningitis, nor would we insist on blood or ascitic fluid or a body-temperature incubator for an organism isolated from the soil. However, let us proceed as though all information were lacking.<sup>1</sup>

**Purification of Culture.**—We must first prove that the culture we are about to study is pure. Microscopic examination of a smear stained by Gram's method may sometimes reveal the presence of

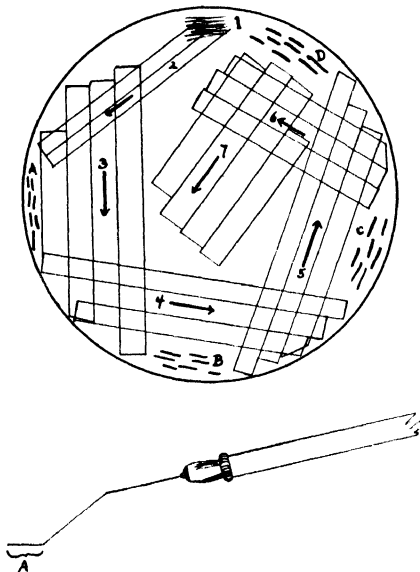


Fig. 112.—Method of streaking a plate so as to secure well-isolated colonies. The original material is deposited at 1. The wire is afterward sterilized in the Bunsen flame. The material is then streaked with the flat part of the wire at 2, 3, 4, 5, 6, and 7, the wire being thrust into the agar to remove excess organisms, as at A, B, C, and D, after each series of parallel strokings. Isolated colonies are almost invariably found at the areas numbered 5, 6 and 7.

The wire used in streaking is shown below. The flat portion at A is brought into contact with the agar from tip to "heel."

contaminants but cannot be depended upon entirely since, as has been pointed out, many different bacteria look and stain exactly alike. The culture must be purified mechanically. This is done by spreading a drop of the material containing the bacteria on some nutrient substance made to resemble as closely as possible the material on which the organism originally grew, if this is known. If not known, autotrophic and heterotrophic media must be fur-

nished, some of the latter with blood, others without. Generally, suitable nutrient mixtures are made solid with agar for this purpose, but slices of potato, coagulated egg, or other solid materials may be used. Petri plates are most convenient. This will permit the growth of isolated colonies much as Koch observed them on his slices of potato. A very useful method of streaking plates is shown in Figure 112. If contaminating bacteria are present, they will probably be evident, after incubation of the plates, as colonies of distinctive appearance.

Let us assume that our organism is heterotrophic and use three plates of blood-meat-infusion agar. Let us also inoculate three plates of extract agar in the same manner. If thought necessary, three silica gel or agar plates may be prepared from some of the solutions noted as serving for the cultivation of autotrophic bacteria, *Azotobacter* or *Rhizobium*, etc. (See section on methods of cultivating bacteria, page 160.)

One plate of each kind of medium may now be incubated at 20° C., one at 37° C. and one at 55° C. (Fig. 113). After 24 hours there may be no growth, in which case we may continue incubation for several days. But let us suppose that there is no growth on the "inorganic" plates, very sparse growth on the extract and infusion plates incubated at 20° C. and 55° C., while good growth occurs on plates of both the extract and infusion media held at 37° C. This, then, tells us the approximate optimum temperature for growth and also gives us an idea as to the kind of media likely to be of use in dealing with our organism. Knowing the pH of the medium, it also gives information on growth requirements with respect to this factor. We will assume that a general-purpose medium with a pH of about 7.6 was used.

An inspection of the growth gives an idea as to the size, shape, color and consistency of colonies. Let us say that the colonies are

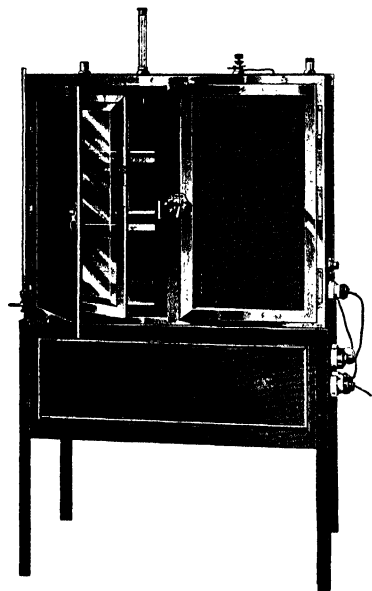


Fig. 113.—Type of bacteriological incubator. The base contains heating units electrically controlled by thermostats in the incubator cabinet.

about 1 to 2 mm. in diameter, glistening, convex, circular, opaque, butyrous (butter-like) in consistency and lemon yellow in color.\*

A convenient way of recording these facts is to use the chart published by the Society of American Bacteriologists, a copy of which is shown here (Fig. 114). By carefully transferring a portion of one of these colonies (Fig. 115), with a sterile needle into a tube of infusion broth (Fig. 116), we provide ourselves with a purified culture, which may be studied further as described below.



Fig. 115.—Subculturing bacteria. Transfer of bacterial colonies from Petri dish to tubes containing culture media. By this method the desired strains of bacteria are isolated. (Courtesy Parke, Davis & Co.)

If no growth occurred on any of the plates inoculated with the original material, we may assume that:

- (a) No living bacteria were present in the inoculum; or
- (b) The temperatures used were not suitable; or
- (c) Some other medium, possibly with a different reaction (*pH*), is necessary; or
- (d) The bacteria may have been strict anaerobes.

Suitable adjustments of conditions must then be made until growth is obtained. Sometimes this is very difficult.

Assuming that good growth was obtained on the plates and

\* *Potato Medium*.—Pigment is often beautifully shown on cubes of potato sterilized with a few drops of water in the bottom of a tube.





Name of organism \_\_\_\_\_ Source \_\_\_\_\_ Studied by \_\_\_\_\_ Culture No. \_\_\_\_\_  
 Date of isolation \_\_\_\_\_ Habitat \_\_\_\_\_ Optimum conditions: Media \_\_\_\_\_ Temp. \_\_\_\_\_ °C  
 Is phase variation observed? \_\_\_\_\_ Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) \_\_\_\_\_ Phases recorded on other charts: \_\_\_\_\_

Underneath required terms		SURFACES		BRIEF CHARACTERIZATION	
<b>VEGETATIVE CELLS:</b> Medium used _____ Reaction (pH) _____ Temp. _____ Age _____ d Size of Majority _____ Ends, rounded, truncate, concave, tapering _____ MOTILITY: In both _____ On agar _____ SPORANGIA AND ENDOSPORES: present, absent _____ Medium used _____ pH _____ Temp. _____ Age _____ d Endospore Form: spherical, ellipsoidal, cylindrical _____ <b>IRREGULAR FORMS:</b> _____ Present on _____ in _____ days at _____ °C		<b>Surface Colonies</b> _____ <b>Deep Colonies</b> _____		<b>As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:</b> U, undetermined; V, variable; X, doubtful. Form & arrangement: 1, streptobacilli; 2, diplococci; 3, micrococci; 4, mucous; 5, rods; 6, comma; 7, spirilla; 8, branched rods; 9, filamentous Diameter: 1, under 0.5µ; 2, between 0.5µ and 1µ; 3, over 1µ Gram stain: 0, negative; 1, positive Flagella: 0, absent; 1, peritrichous; 2, polar; 3, present but undetermined Capsules: 0, absent; 1, present Chains (4 or more cells): 0, absent; 1, present SPORANGIA: 0, absent; 1, ellipsoidal; 2, short rods; 3, spindle; 4, clavate; 5, drumstick ENDOSPORES: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal <b>AGAR STREAKS:</b> Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty Lustre: 1, glistening; 2, dull <b>AGAR COLONIES:</b> Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular Surface: 1, smooth; 2, contoured; 3, rugose <b>GELATIN COLONIES:</b> Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous Surface: 1, smooth; 2, contoured; 3, rugose <b>Biologic relationships:</b> 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic Relation to free oxygen: 1, strict aerobic; 2, facultative anaerobic; 3, strict anaerobic; 4, microaerophile In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent Indole: 0, negative; 1, positive Hydrogen sulfide: 0, negative; 1, positive Hemolysis: 0, negative; 1, positive Methemoglobin: 0, negative; 1, positive <b>PROTEIN LIQUORATION OR DISSOLUTION:</b> Gelatin: 0, negative; 1, positive Casein: 0, negative; 1, positive Egg albumin: 0, negative; 1, positive Blood serum: 0, negative; 1, positive <b>INDICATOR REDUCTION:</b> Litmus: 0, negative; 1, positive Methylene blue: 0, negative; 1, positive Jarrow green: 0, negative; 1, positive Resazurin production: 0, negative; 1, positive	
<b>AGAR COLONIES:</b> Temperature _____ °C. Age _____ d Form, punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid. Surface, smooth, rough, concentrically ridged, radially ridged. Edge, entire, undulate, lobate, even, filamentous, curled. Liquefaction, cap, mass, spreading. Surface, smooth, concave, rugose. Optical Characters, opaque, translucent, opalescent, iridescent.		<b>Surface Colonies</b> _____ <b>Deep Colonies</b> _____			
<b>GELATIN COLONIES:</b> Temperature _____ °C. Age _____ d Form, punctiform, circular, irregular, filamentous. Elevation, flat, raised, convex, pulvinate, crateriform (liquifacient). Edge, entire, undulate, lobate, even, filamentous, curled. Liquefaction, cap, mass, spreading. Surface, smooth, concave, rugose. Optical Characters, opaque, translucent, opalescent, iridescent.		<b>Surface Colonies</b> _____ <b>Deep Colonies</b> _____			
<b>AGAR STREAKS:</b> Temperature _____ °C. Age _____ d Growth, waxy, moderate, abundant, none. Form of growth, filiform, echinulate, beaded, spreading, arborescent, raised. Lustre, glistening, dull. Chromogenesis _____ photogenic, fluorescent. Odor, absent, decided, resembling _____ Consistency, butyrous, viscid, membranous, brittle. Medium, gray, brown, red, black, green, unchanged.		<b>Medium:</b> Temperature _____ °C. Age _____ d			
<b>NUTRIENT BROTH:</b> Temperature _____ °C. Age _____ d Surface growth, ring, pellicle, flocculent, membranous, none. Clouding, slight, moderate, strong, translucent, persistent, none. Sediment, fine, granular, powdery. Odor, absent, decided, resembling _____ Sediment, compact, flocculent, granular, fatty, viscid. Amount of sediment, abundant, scanty, none.		<b>Medium:</b> Temperature _____ °C. Age _____ d			
<b>GELATIN STAB:</b> Temperature _____ °C. Age _____ d Growth, uniform, best at top, best at bottom. Line of puncture, uniform, beaded, pellicle, villous, arborescent. Liquefaction, none, uniform, irregular, uniform. Sediment, none, granular, flocculent, none. Degree of liquefaction in _____ days _____ complete in _____ days _____ Method used _____ Medium, fluorescent, brown, unchanged.		<b>Medium:</b> Temperature _____ °C. Age _____ d			
<b>FERMENTATION</b> Temperature _____ °C.					
<b>Monosaccharides</b>		<b>Disaccharides</b>		<b>Polysaccharides</b>	
Arabinose _____ Mannose _____ Glucose _____ Fructose _____ Galactose _____ Mannose _____ Sucrose _____ Maltose _____ Trehalose _____ Melibiose _____ Cellulose _____		Raffinose _____ Starch _____ Dextrin _____ Glycogen _____ Glycolic acid _____ Arabinol _____ Mannitol _____ Sorbitol _____ Dextrin _____ Salicin _____ Ascorbin _____ Coniferin _____ n-Butyl Gluc _____		Alcohols _____ Glycerol _____ Ethanol _____ Acetone _____ Mannitol _____ Sorbitol _____ Dextrin _____ Salicin _____ Ascorbin _____ Coniferin _____ n-Butyl Gluc _____	
<b>Glucosides</b>		<b>Alcohols</b>		<b>Glucosides</b>	
Medium _____ containing _____ and _____		Medium _____ containing _____ and _____		Medium _____ containing _____ and _____	
<b>Gas in _____ fermentation tube</b>		<b>Am. CO<sub>2</sub> in Eildridge tube</b>		<b>Reaction (pH) after _____ d</b>	
<b>Titrable acidity in ml. of N/10 NaOH</b>		<b>Reaction (pH) after _____ d</b>		<b>Titrable acidity in ml. of N/10 NaOH</b>	

# SUPPLEMENTARY DATA

<b>TEMPERATURE RELATIONS</b> Medium.....pH..... Optimum temperature for growth.....°C. Maximum temperature for growth.....°C. Minimum temperature for growth.....°C. Thermal death point: Time 10 minutes.....°C. Medium.....pH..... Thermal death time: Medium.....pH..... Temp. Time Temp. Time .....°C. ....min. ....°C. ....min. .....°C. ....min. ....°C. ....min. .....°C. ....min. ....°C. ....min. .....°C. ....min. ....°C. ....min.		<b>RELATION TO REACTION (pH) OF MEDIUM</b> Medium..... Optimum for growth: about pH..... Limits for growth: from pH.....to..... <b>RELATION TO FREE OXYGEN</b> Method..... Medium.....Temp.....°C. Aerobic growth: absent, present, better than anaerobic growth, micro-aerophile Anaerobic growth: absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth Additional data:..... <b>MILK</b> Temperature.....°C. Reaction: .....d. ....d. ....d. ....d. Acid curd: .....d. ....d. ....d. ....d. Rumen curd: .....d. ....d. ....d. ....d. Peptonization: .....d. ....d. ....d. ....d. <b>LITMUS MILK</b> Temperature.....°C. Reaction: .....d. ....d. ....d. ....d. Acid curd: .....d. ....d. ....d. ....d. Rumen curd: .....d. ....d. ....d. ....d. Peptonization: .....d. ....d. ....d. ....d. Reduction of litmus begins in.....days, ends in.....days		<b>ACTION ON ERYTHROCYTES</b> Cells..... Method: plate, broth, filtrate Hemolysis: negative, positive Methemoglobin: negative, positive <b>PRODUCTION OF INDOLE</b> Medium..... Test used..... Indole absent, present in.....days <b>PRODUCTION OF HYDROGEN SULFIDE</b> Medium..... Test used..... H <sub>2</sub> S absent, present in.....days <b>ACTION ON NITRATES</b> Medium.....Temp.....°C. Nitrite: .....d. ....d. ....d. ....d. Gas (N <sub>2</sub> ): .....d. ....d. ....d. ....d. Medium.....Temp.....°C. Nitrite: .....d. ....d. ....d. ....d. Gas (N <sub>2</sub> ): .....d. ....d. ....d. ....d. Ammonia production (in amino-N-free nitrate medium): negative, positive Complete disappearance of nitrate in.....medium: negative, positive Disappearance of 2 p.p.m. nitrite in.....medium: negative, positive		<b>REDUCTION OF INDICATORS</b> Medium.....pH.....Temp.....°C. Indicator Conc. Reduction: .....% .....hr. ....d. .....% .....hr. ....d. .....% .....hr. ....d. .....% .....hr. ....d. <b>STAINING REACTIONS</b> Gram: .....d. ....d. ....d. ....d. Method..... Spores: Method..... Capsules: Method..... Medium..... Flagella: Method..... Special Stains:..... <b>ADDITIONAL TESTS</b> Methyl red: negative, positive Voges-Proskauer: negative, positive Growth in sodium citrate: absent, present Growth in urea acid: absent, present Hydrolysis of starch: complete (indies colorless); partial (indies reddish-brown); none (indies blue) Nitrogen obtained from the following compounds:.....	
<b>CHROMOGENESIS</b> Gelatin..... Agar..... Potato..... <b>OTHER PHOTIC CHARACTERS</b> Photogenesis on..... Iridescence on..... Fluorescence in.....							

## PATHOLOGY

### ANIMAL INOCULATION

Medium used..... Age of culture..... Amount..... Incubation period.....

	Whole culture	Cells	Filtrate
Animal			
Type of infection	Subcutaneous		
	Intraperitoneal		
	Intravenous		
	Per os		

\*In each instance where pathogenicity is observed, indicate location of lesion, and type, e. g. edema, histolytic, gas, hemorrhagic, ulcer, diptheritic, etc.

### ANTIGENIC ACTION

Animal..... Medium used..... Age of culture.....  
 Type injection..... Number of injections.....  
 Culture causes production of cytotoxins, agglutinins, proteolytic, emulsin.  
 Specificity: Antibodies produced effective against other antigens as follows.....  
 Immune sera from.....  
 .....effective against this organism as antigen

## SPECIAL TESTS



in the "pure culture," the next step in identification is a study of motility, arrangement, morphology and staining reaction. The two former characters may best be determined by observation in a hanging-drop preparation of the growth in broth. The hanging drop has already been described (see page 44). Let us assume



Fig. 116.—Manipulation of culture tubes, cotton plugs and inoculating needle. The needle, held as shown, is first heated to redness in the Bunsen flame. The two tubes are held almost horizontally in the left hand, the first (nearest the reader) supported by the index finger, and the second by the middle finger, the base of each tube lightly grasped by the thumb. In this way, the operator has an unobstructed view of the contents of the tubes.

The first plug is grasped and removed by the little finger of the right hand, the second plug is grasped and removed by the fourth finger.

After a few seconds are allowed for the needle to cool, it is quickly inserted into one tube, withdrawn and used to inoculate the other. The plugs are then quickly re-inserted in their respective tubes, the needle flamed and put on the desk. Only then are the tubes placed in their rack. If bits of cotton adhere to the mouths of the tubes after withdrawal of the plugs, they may be singed in the flame momentarily. This process does *not* sterilize the mouths of the tubes. It does no more than warm them. Waving the open tubes to and from the flame is not necessary and introduces dust into them. It is, however, a practice generally indulged in.

that our organism is a coccus (spherical or nearly so), although this must be confirmed by microscopical examination of a fixed, stained preparation.

**Arrangement and Motility.**—Cocci are classified according to their arrangement in chains (streptococci), irregular groups

(staphylococci or micrococci) or cubical packets (*Sarcina*) (see page 531). A hanging drop prepared with a *young broth culture* is a useful means of determining how bacteria are arranged. Chains of cocci or bacilli are readily observed, while cubical packets of *Sarcina* can be seen turning over and over like little bundles floating in the medium. *Staphylococcus* and *Micrococcus* groups appear to be very irregular, like bunches of grapes. Motility, if present, is easily seen in hanging drops. Let us assume that our unknown is nonmotile.

**Staining Reaction and Morphology.**—After examining a hanging drop, the next step is to determine the morphology and staining reaction. Gram's stain is of great value, as it not only makes the study of morphology and arrangement more exact but shows us whether we are dealing with one of the great groups of gram-positive or gram-negative organisms. One must bear in mind that Gram's method of staining requires experience and that the differentiations it gives are not absolute. Some organisms are gram-positive only when young or when cultivated on blood or serum media. Let us assume that the organism under discussion is gram-positive.

The morphology of any organism always varies to some extent. A given variety of bacteria may, in any one culture, produce cells of varying size just as, in a group of men or horses, some may be large and some small. If the bacteria are cocci, some of the individual cells may not be perfectly round but some may be oval. If bacilli, some may be long and thin, others short, oval and thick; some may occur singly, others in pairs or chains or long filaments. But the predominating form, size and arrangement of the cells in a pure culture are usually quite apparent and constitute a most important and reliable differential character. Let us assume that, confirming the indications seen in the hanging drop, the smear shows our organism to be a coccus and to have an irregular, grape-bunch-like type of arrangement.\* It seems, therefore, to be a *Staphylococcus* or *Micrococcus*. Such cocci are never motile.

Summarizing our knowledge at this point, we may state that we are dealing with a nonmotile, gram-positive coccus, belonging to the group of staphylococci or micrococci, growing well on plain extract medium as well as blood-infusion medium, at a pH of about 7.4, producing opaque, glistening, lemon-yellow col-

\* Often, in young cultures, the bunches are still small and consist mostly of pairs of newly divided cells. It requires some experience to make the necessary differentiations between the different sorts of coccal arrangements.

onies and preferring a temperature of about 37° C. (body temperature). There is still, however, a good deal to learn about our unknown before identification is complete.

**Biochemical Tests.**—The experienced bacteriologist would know at once exactly what peculiarities to look for in dealing with a culture of gram-positive cocci which have the characters we have enumerated. For the present, however, we shall put ourselves in the place of a person to whom a gram-positive coccus is a new and hitherto unknown plant.

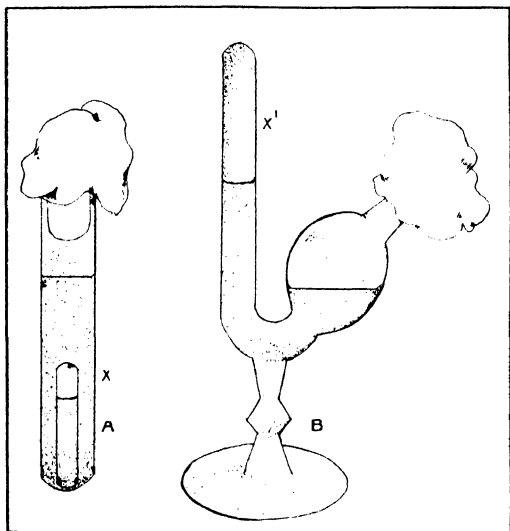


Fig. 117.—Types of apparatus used for collecting gas produced by bacteria. A, Durham tube (gas has collected at X). B, Smith tube (gas has collected at X').

Since certain enzymes are highly characteristic of bacteria, the power of our unknown organism to ferment or hydrolyze some of the carbohydrates commonly in use, such as dextrose (glucose), saccharose (cane sugar), and lactose (milk sugar), should be tested, as well as its power to hydrolyze or attack such familiar nitrogenous and protein substances as sodium nitrate, sodium nitrite, gelatin, coagulated serum, the casein in milk, etc.

**Fermentation Tests.**—These tests may be made as described on page 157. When dealing with an unknown, each tube of broth should contain a small inverted vial (placed there *before* sterilization) (see Fig. 117) to catch any gas that may be formed. Gas

might otherwise pass off into the atmosphere and not be detected. Two organisms, both of which ferment the same carbohydrate, may be sharply differentiated if one forms gas while the other does not.

When gas is produced from carbohydrates by bacteria, it is *prima facie* evidence of fermentation and is always accompanied by souring or acid formation. However, fermentation often occurs

#### FERMENTABLE SUBSTANCES USED IN BACTERIOLOGY

##### *Monosaccharides*

###### Pentoses:

l-arabinose  
xylose  
rhamnose

###### Hexoses:

dextrose  
d-mannose  
galactose  
d-leulose

##### *Disaccharides*

sucrose  
lactose  
trehalose  
cellobiose

##### *Trisaccharides*

raffinose  
melezitose

##### *Polysaccharides*

inulin  
starch  
glycogen

##### *Alcohols*

Trihydric:  
glycerol

###### Tetrahydric:

d,l-erythritol

###### Pentahydric:

adonitol

###### Hexahydric:

d-manitol  
dulcitol  
sorbitol

##### *Glucosides*

salicin  
esculin  
arbutin  
phloridzin  
convallamarin  
colocyntin  
amygdalin  
 $\alpha$ -methyl-glucoside

##### *Organic esters*

sodium hippurate  
sodium citrate  
sodium formate  
sodium succinate  
sodium tartrate

##### *Other substances*

inositol  
perseit  
d-glucosamine (HCl)

without gas production, and then acid formation is our only evidence that the organism has metabolized the carbohydrate. Acid may be detected by adding to the medium an indicator or dye such as bromcresol purple which changes color in the presence of acid. The change in color of the indicator is our proof of fermentation. However, as has been pointed out, some species can metabolize these acids, so that observations should be made every 24 hours in

order that the culture may not revert to an alkaline reaction before the acid formation has been noted. Variant strains of the same species may differ considerably in the *rate* at which these reactions are brought about and ample time for observation must therefore be allowed.

Some of the substances frequently used in studying the fermentative powers of various organisms are listed on page 226.

**Gases Produced by Bacteria.**—The nature of the gas formed by bacteria may be of great differential value. As shown in the sections on metabolism, etc. (page 351), two gases commonly given off by bacteria are carbon dioxide and hydrogen in various proportions. It is often of the greatest importance, especially in sanitary bacteriology, to determine the ratio of carbon dioxide to hydrogen. This is described more fully in the section dealing with water bacteriology (page 438).

Carbon dioxide and hydrogen are not the only gases produced by bacteria, however. Many organisms, in their metabolism of proteins or protein-digestion products, *e.g.*, cystine, taurine and other sulfur compounds, set free hydrogen sulfide, often in large amounts. This gas is one of the most noticeable in connection with putrefactive processes. Some organisms may be identified or differentiated from others by their power to produce  $H_2S$ . Thus, *Salmonella schottmülleri* produces hydrogen sulfide in its decomposition of amino acids, while *Salmonella paratyphi* does not.

*Hydrogen sulfide* is readily detected in cultures by the use of lead acetate which reacts with  $H_2S$  to form black lead sulfide. The acetate may be added directly to the culture medium, or, it may be suspended above the medium in the tubes on strips of sterile filter paper impregnated with the salt, held between the glass and the cotton plug.

Methane is another gaseous product of bacterial metabolism. It results usually from the fermentation of cellulose, especially under anaerobic conditions. In swampy places anaerobic bacteria attack the cellulose of dead vegetation and sometimes large amounts of the gas are given off. The bubbles seen arising during the summer time in woodland swamps are nearly always methane, although hydrogen and hydrogen sulfide may also be present in small amounts, as well as carbon dioxide. *Clostridium butyricum* and similar anaerobes are active in this respect.

Some organisms produce poisonous gases under certain circumstances. For example, *Pseudomonas aeruginosa* is said to produce hydrogen cyanide (HCN), while a number of species of *Coryne-*



*bacterium*, especially *C. diphtheriae*, produce hydrogen telluride when cultivated, as is frequently done, upon medium containing potassium tellurite. Many bacteria, especially saprophytic species, also produce large amounts of ammonia and nitrogen during protein decomposition.

**Proteolysis.**—In addition to making tests with fermentable substances, tubes containing common proteins (nutrient gelatin, coagulated serum and sterile milk) may be inoculated to deter-

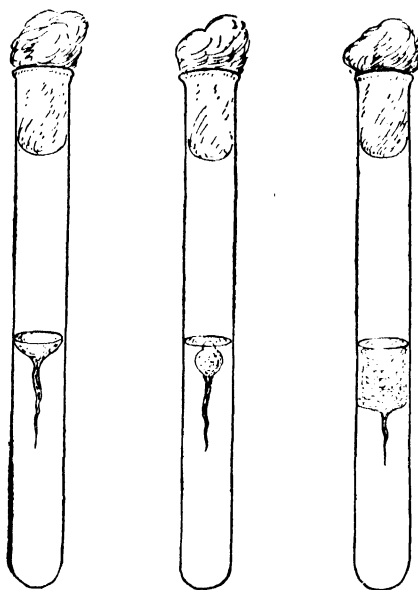


Fig. 118.—Types of gelatin liquefaction by bacteria. (Zinsser and Bayne-Jones: "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

mine the ability of our unknown to attack different proteins. These may be incubated with the carbohydrate tubes.

**Gelatin.**—A tube of solidified nutrient gelatin is inoculated by "stabbing" a wire, having the desired bacteria upon it, down into the depths. Many workers prefer to incubate the gelatin at 20° C. rather than at body temperature. At the former temperature the gelatin remains solid except where digested by the organism, and the shape of the portion liquefied may be observed (Fig. 118). This was formerly regarded as of great significance, but is really of much less value than other tests. The time lost in waiting for

growth to occur at 20° C. (if indeed it occurs at all) is much more valuable than the information as to form of the area liquefied. Gelatin cultures held at 37° C. liquefy completely due to the temperature but may be placed daily in the refrigerator for a sufficient time to allow them to solidify if undigested. This should be continued for 2 weeks unless evidence of digestion is obtained sooner. An uninoculated tube of gelatin is incubated with the others to serve as a guide in refrigeration time. Those tubes in which the gelatin fails to solidify may be marked "+" or "digested." *The tubes should not be shaken while warm, as growth, and a small amount of gelatin digestion, may at times occur only in the surface layer and this would be masked were it mixed with the bulk of the warm, fluid culture.*

*Serum Digestion.*—Coagulated serum may be prepared by mixing three parts of horse or beef serum with one part of nutrient broth. Glucose is sometimes incorporated in a concentration of 0.25 percent. This is Löffler's medium so commonly used in the study and diagnosis of diphtheria. The reaction needs no adjustment if the serum is fresh. Five-cc. amounts of the mixture are placed in tubes which are then put in the autoclave in a slanting position and heated at 100° C. or more for an hour. The serum is coagulated and sterilized at the same time. The slants are inoculated when cool, by smearing a loopful of broth culture or growth from agar over their surfaces.

When attacked by bacteria the serum usually becomes brownish and translucent and the growth appears to sink inward. Total liquefaction sometimes occurs in 48 hours at 37° C., especially in cultures of aerobic spore-forming bacilli, but is often delayed for as long as 2 weeks.

*Action on Milk.*—Milk is rich in nutrient substances, containing casein, fat, mineral salts, lactose, vitamins and other materials. It has a pH of around 6.8 when fresh. It is an ideal culture medium for many bacteria, especially those adapted to growth in body fluids, *i.e.*, parasites like *Eberthella typhosa* and *Corynebacterium diphtheriae*. Its sanitary relationships thus become clear.

For use as a bacteriological culture medium, skimmed milk is tubed in 5-cc. amounts and sterilized by tyndallization or autoclaving. It furnishes an excellent test medium. If an indicator, such as litmus or bromocresol purple, be added, *fermentation* of the lactose may be detected. In addition, *rennet production* may be inferred if the milk is curdled (provided this is not due to souring; a point difficult to determine if fermentation occurs). *Digestion*

of the casein often follows coagulation. The milk then becomes brownish and translucent and the clot disappears.

**Other Proteins.**—In addition to milk, gelatin and serum, other protein substances such as cooked egg and ground-up beef are sometimes used for studying bacteria. In fact, one may use any protein his ingenuity suggests. In all tests upon proteins and protein-decomposition, carbohydrate-free media should be used (see section on “protein sparing action” of carbohydrates, page 354).

**Fibrin.**—For example, Tillett and Garner<sup>2</sup> have developed a method of testing certain specific proteolytic activities of pathogenic streptococci. These organisms, especially the human pathogens, possess the power of digesting the fibrin clot of human blood in a short time.\*

**The production of ammonia** from the deamination of amino compounds and from other biochemical reactions is of great value in certain bacterial differentiations. This may be detected by inserting a moistened strip of red litmus paper between the cotton plug and glass tube of a culture so that ammonia fumes arising from the culture will affect the litmus. The litmus may also be affected by volatile amines, so that the test is not wholly accurate.

**Bacterial Reductions.**—In addition to tests with carbohydrates and proteins, other features of the physiology of the organism may be studied. A physiological property characteristic of many bacteria is the power to abstract oxygen from various substances. This power of reduction is found in many species and indicates an ability to obtain and utilize oxygen from sources other than the atmosphere. It is a very useful differential character.

**Nitrate Reduction.**—One method of determining the power of reduction is to incubate the bacteria being investigated in broth containing about 0.1 percent of sodium nitrate ( $\text{NaNO}_3$ ). After

\* Fibrinolysin test (Tillett and Garner):

1. In a test tube place 0.2 cc. of fresh (not over 48 hours old) oxalated human plasma. (Oxalated plasma is prepared by drawing 10 cc. of blood into a tube containing 0.02 gm. of sodium oxalate, or 1 cc. of 2 percent oxalate. Centrifuge and use the clear supernatant fluid. Keep cold.)
2. Add, successively:
  - (a) Normal saline solution, 0.8 cc.
  - (b) Broth culture to be tested (24-hour-old, well-grown culture is necessary), 0.5 cc.
  - (c)  $\text{CaCl}_2$  (0.25 percent in 0.85 percent  $\text{NaCl}$ ), 0.25 cc.
3. Mix and immediately place in water bath at  $37^\circ\text{C}$ .
4. Observe the time of solid coagulation, and then measure the interval required for the clot to be liquefied. This may vary from 15 minutes to several hours.

48 hours and at other intervals, a test is made for the presence of *nitrites* ( $\text{NaNO}_2$ ) by withdrawing a little of the culture from the *bottom* of the tube and immediately adding a few drops of sulfanilic acid solution\* and a few drops of dimethyl-alpha-naphthylamine solution† or by allowing the drops to settle to the bottom of the whole culture.

The development of a red color denotes the presence of nitrites, but the failure of this color to develop raises a question. Either (a) the nitrate has not been attacked, so that no nitrite is present or (b) nitrite has been formed but also attacked and reduced to free nitrogen or ammonia. A test to see whether any nitrate remains may be made by adding a little pulverized zinc. This reduces any remaining nitrate to nitrite (which may be tested for as above) and tells us whether or not the organism has utilized *all* of the nitrate. If this test is positive (*i.e.*, nitrate is still present), the original test for nitrites having been negative, then it is clear that the organism did not attack the nitrate at all. If both the nitrate and nitrite tests are negative then it is obvious that the organism utilized all the nitrate as well as nitrite.

**Nitrite Reduction.**—The latter point, *i.e.*, ability of the organism to attack nitrite, may be determined separately by testing the ability of the organism to destroy the nitrite in cultures known to contain it. This is done by incubating cultures containing quantities of nitrite so small (about 0.002 per cent) as just barely to give a positive test for nitrites. If the organism is capable of reducing nitrite, the culture will soon lose its power of reacting positively to the nitrite reagent, because the organism will quickly reduce all of such a small amount of nitrite.<sup>4</sup> A sterile control tube should be tested at the same time, as illuminating-gas fumes often contain nitrous acid which may give a slight reaction in such tubes.

**Reduction of Litmus.**—The ability of an organism to reduce other substances than nitrates and nitrites is often investigated. Litmus, for example, is often used as an acid-indicator in milk cultures, but it also serves to show whether or not the organism has strong reducing powers by becoming entirely decolorized when

\* Sulfanilic acid solution:

Glacial acetic acid . . . . .	100.0 cc.
Water . . . . .	250.0 cc.
Sulfanilic acid . . . . .	2.8 gm.

† Dimethyl-alpha-naphthylamine solution (Wallace and Neave<sup>3</sup>):

Glacial acetic acid . . . . .	100.0 cc.
Water . . . . .	250.0 cc.
Dimethyl-alpha-naphthylamine . . . . .	2.1 cc.

reduced. Just enough is added to the medium before sterilization to give a definite color.

**The Reductase Test.**—Methylene blue is similarly decolorized by many organisms. The dye is used as a hydrogen acceptor in respiration (see section on bacterial respiration, page 363). Many other compounds are similarly utilized. Standardized solutions of methylene blue are often added to milk samples to estimate, roughly, whether a few, a moderate number, or enormous numbers of bacteria are present. When great numbers are present, the blue color may disappear almost immediately; when less, it may remain unchanged for two hours or more. Such a test is known as a methylene blue reduction test or, since the reduction was thought to be due to an enzyme called reductase, the *reductase test*. The rate of reduction also depends on the kind of bacteria present, other hydrogen acceptors present in the solution, availability of oxygen, etc. (see section on bacteria in milk, page 564).

**Indol.**—Indol is a substance resulting from the attack of bacteria upon the amino acid *tryptophane* (see section on metabolism, page 355). It is usually tested for in cultures made in broth prepared with peptone containing tryptophane. Cultures are incubated for 48 to 72 hours. Indol reacts with acidified solution of Ehrlich's reagent (para-dimethyl-amido-benzaldehyde)\* to produce a pink compound.

The culture to be tested should be shaken with 1 cc. of xylol. Indol is soluble in xylol and is concentrated in it and carried to the surface by the solvent after a minute of standing. A few cubic centimeters of Ehrlich's reagent are then *gently* added to the culture and are made to remain in a layer between the xylol and the medium. If indol is present a pink color forms in a few minutes as a "ring" at the junction of the xylol and the reagent. Pink compounds *not* soluble in xylol sometimes develop in the presence of Ehrlich's reagent if the xylol is not added first. These are not due to indol.

**Identification of the Unknown Organism.**—Tubes of test media of our unknown organism, prepared as above described, are inoculated from the purified culture of our organism and are incubated as stated (37° C.) and carefully observed until all changes have ceased and all tests have been completed. An incubation of 4 to 5

\* Ehrlich's reagent:

Ethyl alcohol (95%) . . . . .	380 cc.
HCl (conc.) . . . . .	80 cc.
Para-dimethyl-amido-benzaldehyde . . . . .	4 gm

days is usually sufficient. A tabulation is then made of the results. For the organism under investigation, let us assume that they are as follows:

Pigment (as observed in colonies on agar)—lemon yellow.

Lactose—fermented; no gas.

Dextrose—fermented; no gas.

Saccharose—fermented; no gas.

Salicin—not fermented.

Gelatin—slowly liquefied.

Agar slant and cubes of potato—good growth, soft, moist and glistening, lemon yellow in color.

Broth—turbid, sediment is yellow; faint pellicle (scum).

Milk—coagulated, acidified.

Nitrates—reduced to nitrites.

Indol—not produced.

How are we now to determine the genus and species of the gram-positive, irregularly arranged cocci which we have been studying?

**Use of Keys.**—Although every experienced bacteriologist has at his finger tips, so to speak, all of the distinguishing cultural reactions and other identifying characters of the organisms with which he is working, it is unusual, to say the least, to find one who knows *all* the characters of *all* the species. When an unknown organism is encountered which must be identified, the main morphological and tinctorial features are determined in some such manner as just described and then recourse is had to *keys* or other reference works. Probably the most useful key for general bacteriological use is "Bergey's Manual of Determinative Bacteriology."<sup>5\*</sup>

**Genus and Type Species.**—There are certain central types of bacteria as, for example, *Streptococcus pyogenes*, *Bacillus subtilis*, *Clostridium butyricum*, and the like. Each of these is a well-known, thoroughly studied, easily identifiable species representative of a genus or a group of species, and is spoken of as the *type species* of that genus or group of organisms. To every experienced bacteriologist each of these names conveys a very definite idea as to the characters of the group. Each type species is a beacon, as it were, in a confused sea of similar but often illy defined, partly studied organisms frequently distinguishable from the central or type species of the genus or from each other only with the greatest

\* All names of bacteria given in this book are based on the 1939 edition (the 5th) of "Bergey's Manual" unless otherwise noted.

difficulty or not at all, or, on the other hand, differing so markedly from the type species that the relationship seems very tenuous. To attach a species name to each of the similar types puts as much emphasis upon it as though it, too, were as fully known and studied as the type species, and this confuses values. If, for example, we examine the genus of which *Bacillus subtilis* is the type species, we find the names of over 150 organisms which are mostly gram-positive, aerobic, motile, spore-bearing, saprophytic, straight rods. Many of them were incompletely studied by the person who named them originally, so that often only one or two points concerning them are given. Furthermore, these points are often subject to correction by later workers, and the organisms designated may turn out not to be new species at all. Examine the list of names at various times attached to *Bacillus cereus* or to *Bacillus mesentericus*, *Clostridium butyricum* or *Streptococcus lactis* for examples. There are numerous others. But let us return to our "unknown" and the use of "Bergey's Manual." We should first determine to which of the 7 orders of the class Schizomycetes our culture belongs. On pages 69 and 70 of the Manual is to be found a brief synopsis of the characters used to differentiate the 7 orders. Obviously the species in question is neither plantlike nor moldlike (Order II); is not stalked, sheathed, or alga-like (Order III or IV); or full of sulfur granules (Order V); neither is it slimy (Order VI); nor protozoan-like (Order VII). We are thus left to consider Order I, the true bacteria or Eubacteriales.

On pages 70 and 71, we find a synoptic description of the families of the order Eubacteriales. Our organism is spherical and may therefore fall into either family I (Nitrobacteriaceae), family VI (Micrococcaceae), family VII. (Neisseriaceae) or family IX (Lactobacteriaceae). It is obviously not a member of the other families, which include only rod-shaped or filamentous bacteria. To settle the choice between families I, VI, VII and IX, more exact information is necessary and we turn to page 72. On page 72 *et seq.*, we find that in only one genus of family I, the *Nitrosococcus*, are the organisms spherical. But the organisms of this genus do not grow on ordinary heterotrophic culture media, whereas our "unknown" does. This, therefore, leaves us to consider families VI, VII, and IX and to them we turn our attention.

Reference to page 278 makes it clear that we are not dealing with Neisseriaceae (family VII) since they are gram-negative; and we eliminate at present also the Lactobacteriaceae (page 315) (tribe Streptococceae) since our organisms grow well on ordinary

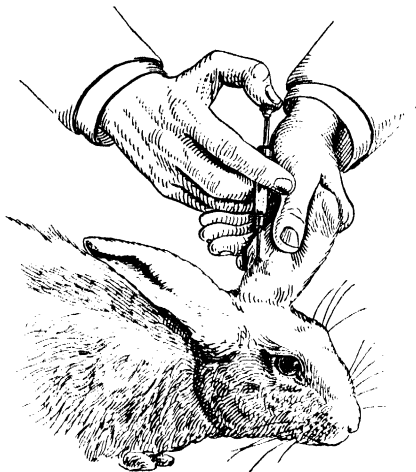


Fig. 119.—Injection of bacteria into a rabbit through a vein in the ear. (McFarland.)



Fig. 120.—Method of inoculating a guinea pig with microorganisms: this injection is into the peritoneal cavity. (Therapeutic Notes, October, 1942, Parke, Davis & Company.)



extract agar without blood and do not occur predominantly in chains but in irregular clumps and masses as well as in pairs.

Family VI (Micrococcaceae), therefore, would seem to be our objective, and we are referred to page 236. Here we find that the descriptions of genera III and IV (*Gaffkya* and *Sarcina*) do not correspond with the organism in question, and that we must search in genera I and II. Our choice, therefore, lies between the staphylococci and the micrococci. The organisms of these two genera resemble each other so closely that it is necessary to consider carefully the characters of the individual species in each group.

A short study of the data we have already obtained by our cultural tests shows that our organism corresponds closely with the description of *Staphylococcus citreus* (page 263), since it produces a lemon-yellow pigment, liquefies gelatin, ferments lactose, grows on potato with lemon-yellow pigment, reduces nitrates to nitrites and fails to produce indol.

A further check upon the identity of the culture may be made by testing other fermentable substances and comparing various characters of organisms closely resembling it, such as *Micrococcus flavescens*. A few repetitions of the tests usually serve to confirm the diagnosis, or prove it to be in error, necessitating further study.

Pathogenicity may be determined by injecting 0.5 cc. of a 24-hour broth culture intravenously and subcutaneously into a rabbit or guinea pig (Figs. 119 and 120). If our unknown is truly pathogenic, abscesses will probably form or the animal may die. Some strains are more pathogenic than others. Further details concerning staphylococci and micrococci will be given when these genera are taken up specifically later (see page 531).

A number of other biochemical tests used principally for certain groups of bacteria will be detailed in the discussions of those organisms and their properties, farther on in this book.

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## CHAPTER 12

# PROBLEMS AND PHENOMENA OF BACTERIAL GROWTH

## BACTERIAL REPRODUCTION

**Binary Fission.**—In all biological reproduction, we have an unsolved mystery. "We should remember, . . . how thickly beset we are with unsolved problems. . . . What, for instance, is the commonest and most universal vital event? It is cell division. And though literature about it would fill a large library we do not yet know the conditions of its occurrence or the forces at work in its accomplishment!" (Thomson<sup>1</sup>). We know that cell reproduction occurs, but how or why we cannot say.

Reproduction by the organisms of the class Schizomycetes (*bacteria* or *fission fungi*) is chiefly by means of transverse division of each cell into two equal cells. This is called simple or *binary fission* (Fig. 121). In bacteria, since we have no definite knowl-



Fig. 121.—Diagrammatic representation of binary fission in a coccus and in a bacillus.

edge of how nuclear material is arranged inside the cell, nothing is known of mitosis in these organisms. Hereditary characters must be maintained by some unknown but probably much more primitive mechanism than chromosome partition.

**Bacterial Multiplication by Means Other than Binary Fission.**—Other forms of multiplication of bacteria, especially by means of intracellular bodies, variously called gonidia, microcysts and regenerative granules, have been described. In these types of reproduction, multiplication may be regarded as resulting from divisions of the cell material which occur without the participation of the cell wall, so that all of the new organisms are contained within the original cell wall and are necessarily very tiny.

There is no convincing evidence that such minute bodies occur in relation to the reproduction of bacteria. In attempting to demonstrate them, one is very apt to be misled by the presence of volutin (food) granules, fat droplets and, in dead or dying cells, of

globules of coagulated protein. Diffraction and refractive effects are much enhanced in bacteria and minute granules due to the acute curvature of their minute surfaces. These optical effects are undoubtedly a source of error in such studies.

The minute bodies are said by some authors to be filtrable, and to represent a transition form between bacteria and the true filtrable viruses. Two questions arise. First, do these minute bodies occur? Second, what is the evidence of their relation to viruses? These problems are discussed more fully in the chapter on viruses (see page 739).

*Sexual Multiplication of Bacteria.*—The question of the existence of sexes among bacteria is a much debated one. One searches in vain for really confirmed proof of the existence of sexual phenomena. Space does not permit an extended discussion of the question here, although it is one of much interest among bacteriologists. Morphological evidence of the existence of sexes, as inferred from the apparent fusion of cells, the formation of protrusions and of bodies resembling zygospores and so on, is very unreliable because of various optical limitations of microscopes, even electron microscopes. Such protrusions and zygospore-like bodies undoubtedly appear, but there is no necessity for regarding them as sexual or reproductive bodies. Further, from a biological point of view, sex in bacteria, if present, would be an anachronism unbelievable in so highly methodical and consistent a process as organic evolution. According to Coulter, "... sex is not an essential feature of reproduction [in the plant kingdom]. Historically it was the last method of reproduction attained among plants, and when it appeared it did not replace older methods [fission] but was added to them. . . . in the evolutionary sequence of plant groups the sexual cells appear for the first time far above the most primitive known plants . . . [bacteria]. . . . the beginnings of sexual cells are seen among both the green algae [as *Ulothrix*] and the brown algae . . ."<sup>1a</sup>

*Reproduction by Budding.*—Budding is really a modified form of fission in which the daughter cells are of smaller size than the parent. In true budding, as exemplified by yeasts, a single cell may give off several daughter cells which may often be seen clinging for some time to the parent cell. Reproduction by the process called budding, if it occurs in *true* bacteria (order Eubacteriales), is probably rare and of a much more primitive nature than true budding as seen in yeasts and higher plants and animals. Possibly some of the protrusions, lumps and bumps observed in bacteria

and already referred to in the preceding paragraph as possible zygosporos, may represent primitive attempts at budding. However, in one of the alga-like orders (Chlamydobacteriales, commonly known as "iron-bacteria") of the class Schizomycetes, which are not classed as *true* bacteria, reproduction by a process very much like budding does take place. Among the Actinomycetales reproduction by conidia and by fragmentation of filaments occurs, much as in molds. These will be discussed in detail farther on.

### BACTERIAL "POPULATIONS"

In soil, river water, feces, saliva and many other substances, experience has led us to expect certain numbers and kinds of bacteria which are always there. We might find 2 million bacteria per gram of garden soil, 10 to 5000 bacteria per cc. of river water, and so on, with certain types normally predominating, others rare, depending on the environment. This is called the *normal flora* of that environment or substance.

An extremely important point to bear in mind in this connection is that, in nature, pure cultures rarely occur. There is a struggle for existence between the various species of bacteria in the soil or on a decaying potato just as there is among fish in the sea or animals on the land. Each is constantly exposed to the effects of the multiplication of other organisms, both plant and animal, as well as to constant changes in temperature, water supply, abundance of nutriment, hydrogen ion concentration, light, air, oxygen, and the like. In the case of bacteria, the difference between such a natural environment and that furnished in the laboratory in test tubes with only one kind of bacterium present, and with constant conditions of temperature, optimum nutriment, pH, and other factors, is vast indeed. One of the very serious drawbacks of pure-culture bacteriological methods is that we never observe the organisms in a truly "natural" environment. However, in many cases we approach natural conditions in some degree.

The numbers of bacteria present in any sample of material depend on a variety of factors, some of which are fairly well understood. For example, we know something of the rate of growth of many species, and some of the factors which affect their predominance in or disappearance from various situations. A good way of clarifying our ideas concerning these matters is to consider an hypothetical bacterial population from the moment of its beginning until the death of the last survivor.

As an illustration we may select a culture flask containing 50 cc. of sterile infusion broth.

**The Colony Count.**—Let us introduce, by means of a sterile wire needle, or a pipette, 10 cells of a common bacterium of the intestinal tract known as *Escherichia coli*. Let us assume that these are from an inactive or “dormant” stock culture. The culture is held at



Fig. 122.—Quebec colony counter. (Courtesy of Spencer Lens Co.)

35° C. The problem before us is to measure the population increase and determine its rate after different intervals of time by counting the numbers of living cells\* present. This may be accomplished in the following manner.

\* Methods will be described later for enumerating the *total* numbers present without differentiation between living and dead cells.

At any desired moment, exactly 1 cc. of the culture is withdrawn from the flask with a sterile measuring pipette and is transferred to a sterile Petri dish. Immediately afterward, about 15 cc. of nutrient (meat extract) agar, previously melted (and cooled to about 40° C. so as not to kill the bacteria), are poured into the dish and the culture thoroughly mixed therewith by a gentle horizontal rotation and oscillation of the dish. In a few minutes the agar will have re-solidified. This plate-culture is held in an incubator at about 35° C. for 24 hours and is then examined for the presence of colonies,\* distributed throughout the agar (Fig. 122). Each of these represents, *theoretically*, the progeny of a single cell which was in the original inoculum and was imprisoned in or on the agar at that point. Actually, several organisms if stuck together in a clump will give rise to only a single colony so that the colony count does not give a wholly accurate enumeration of the live, *individual* cells present in the material under investigation. However, the errors in the plating method are known, in a general way, and within its limitations the plate count is one of our most useful means of enumerating bacteria. It is very widely used and should be fully understood at this point.<sup>2</sup>

**Determination of Growth Curve.**—Let us suppose that we make plate counts on our *Escherichia coli* culture at rather frequent intervals (2 hours) at first, and plot the numbers of colonies (roughly, live organisms per cc.) in our plates against time. We may continue this until no further significant changes occur, and at the end of this time a curve will have been obtained which will look somewhat like that seen in Figure 123.

A totally different type of curve would be obtained if we were to count the bacteria in the fluid by means of a direct microscopic method such as the Breed smear (see page 562) or by the use of a blood-cell counting chamber. The eye in either method would fail to distinguish between live and dead cells and the total number of cells would increase in breath-taking bounds at first so that the first part of the curve would slope very steeply upward. As growth rate slackened, the curve would still slope upward but less steeply.

\* It is often desirable to examine the plate by means of a special illumination and a 2 × or 3 × magnifying glass so as to make even the smallest colonies visible. A convenient device for this purpose is seen in Figure 122. The criss-cross lines are for the purpose of guiding the eye in making the colony count.

As the number increases toward several millions per cc., the 1 cc. of culture removed for the plate count is diluted so that plates are obtained which show only about 50 to 300 colonies. This is easy after a little experience. The number of colonies, multiplied by the dilution, gives the "number per cc."

Only when all growth ceased would the curve reach a level. It would not decline unless bacteria were actually destroyed faster than they multiplied. Destruction of dead bacteria by self-contained enzymes occurs in some species such as pneumococci and gonococci. The process is called *autolysis*.

The growth curve is of interest in that it is representative of all normal population curves, plant, animal and human. By a normal curve is meant one which is not disturbed by special factors such as excessive immigrations or emigrations, wars or natural catastrophes. The population curve of the United States (Fig. 124) has a

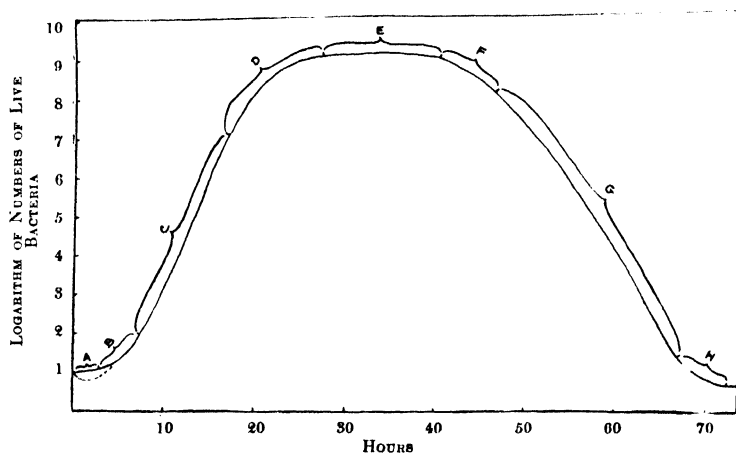


Fig. 123.—Growth curve of hypothetical culture of *Escherichia coli*. For explanation see text.

similar form, but there are factors, such as wars, which cause irregularities to appear from time to time. However, it must be pointed out that the curves shown in Figures 123 and 124 are not strictly comparable, since in the former *logarithms* are plotted against time while in the latter actual *numbers* are used. Were the same system used in Figure 123 as in Figure 124, Figure 123 would have to be some 1200 miles long to accommodate the curve, and it would go up and down precipitously as compared with the curve in Figure 124, since one is scaled in hours, the other in periods of 20 years. Nevertheless the same general principles underlie the form of both curves. The interesting question arises as to whether, in a few generations, the upper level or declining phase in the growth curve of the population of the United States will have been reached.

Will the laws of biology which apply in plant and animal populations govern human populations? They have not yet failed to do so. Wars may make a dent in the curve, but do not change its general course.

**Phases of the Growth Curve.**—The curve shown in Figure 123 has several portions which deserve some brief discussion. These are shown by brackets and labels in the figure. Portion *A*, usually called the *initial stationary phase*, represents a period during which the dormant organisms are probably imbibing water, becoming adjusted to the new environment, and so on, much as might occur

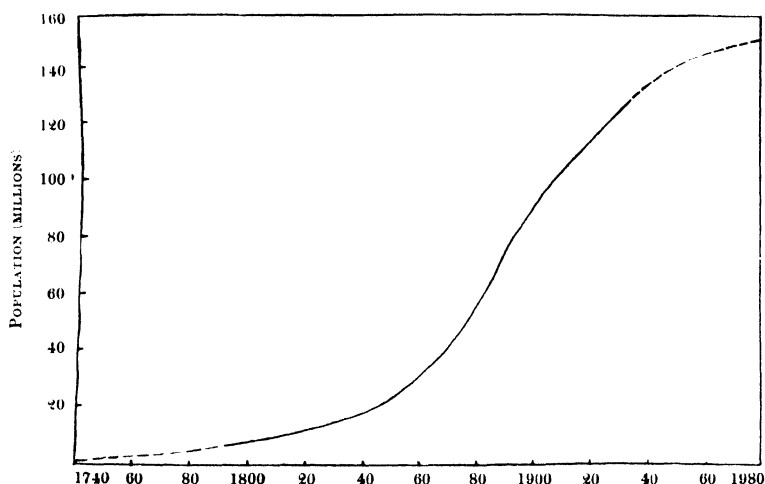


Fig. 124.—Approximate population curve for the United States. Compare with the first portion of the curve for a bacterial population. What will happen to the U. S. population curve by A.D. 2000? Will a war affect it?

when a dormant tree is set out in the spring. There is no increase in numbers. The dotted line indicates that some few of the cells may actually die off during this period, only the more vigorous going on to multiplication which soon begins to show itself by the rising inflection in portion *B* which is usually called the *phase of accelerated growth*.

When very small inocula of certain species are transferred to media like pure serum or egg, consisting of native proteins only, with none of the simpler products of protein hydrolysis present which can diffuse into the cell, the cells cannot initiate growth, for they have nothing but unavailable, undigested proteins on



which to grow. In the absence of growth, they cannot form the necessary proteolytic enzymes. If larger inocula are used, some small amounts of protein hydrolysis products, or of the enzymes themselves, are carried over from the previous culture and allow a few cells to start growth, thus producing enzymes which hydrolyze the native proteins and permit further growth. Also, in the larger inocula, there may be enough dead cells so that, as they disintegrate, sufficient enzyme is produced to initiate growth. This may, in part, explain the lag phase of growth. The mutual stimulation of a number of cells to growth has been called "allelo-catalysis."

During the phase of accelerated growth the time required for each cell to divide is decreasing, fission occurring more and more rapidly as the organisms become adapted to the new culture medium. Eventually they reach their maximum rate of fission, which may become so rapid that the number of organisms doubles every 20 to 30 minutes. Fission rate varies very greatly with different species and under different conditions of growth. Tubercle bacilli, for example, probably divide only about once a day at the highest rate of growth. The logarithms of the numbers of live organisms plotted against time produce a straight line, as shown in Figure 123. This period is spoken of as the *phase of logarithmic increase*. Were this to continue uninterrupted, the culture would become a solid mass of bacteria in a few hours. A single cell of *Escherichia coli* allowed to continue such growth for a year or so, would produce a mass weighing more than the sun!

Fortunately, within a few hours after the commencement of the logarithmic phase, the organisms begin to encounter difficulties. Food begins to run out, poisonous waste products accumulate, and the cells become so crowded together that they jostle one another and this interferes with their proliferation. The rate of fission begins to decline and the older organisms die in increasing numbers, so that the increase in number of live cells slows, as shown in the portion of the curve labeled *D*. This is spoken of as the *phase of negative growth acceleration*.

**Immortality (?) of Bacteria.**—It is interesting to speculate upon the relationships between the age of cells, their multiplication and their continued existence without fission. Let us compare the fate of two cells just produced by the fission of their "parent." Under favorable conditions one undergoes certain unknown physical and chemical changes which are the equivalent of "maturation" and, after a time, undergoes fission like its predecessor. What is the age of the new daughter cells? Are they, physiologically, infantile cells, or have they the age of their parent cell? What mechanism determines when they shall again undergo the mysterious

changes of maturation and in turn become young again by the process of fission, thus theoretically propagating indefinitely and never growing old or undergoing senescence? Are they, by virtue of continual renewal of youth, immortal?

The other of our two original cells fails to reach a state in which fission occurs, and remains intact. What is its physiological age status? If transferred to a new culture it may begin multiplication at once and become "young" or it may die. It may die even if left in the original culture. Does it die of "old age"? The explanation of its fate is quite obscure. Evidently chronological age and physiological age may be very different matters among bacteria.

**Final Phases.**—Eventually (the time depending on the temperature, the size of the flask and volume of fluid, the composition of the medium and numerous other factors), the number of cells dying balances the rate of increase, and the total population remains unchanged for a time. This phase, the *maximum stationary phase*, is shown at *E*.

As conditions become more and more inimical to the bacteria, they grow more slowly, and death overtakes them in ever-increasing numbers, as shown at *F*, the *phase of accelerated decrease*.

Th's progresses into the *logarithmic death phase* (*G*), during which decrease in number occurs at a regular, unchanging rate.

Finally, conditions begin to reach an equilibrium such that both rate of death and rate of increase tend to balance each other again at a very low population level, and the *phase of readjustment* (*H*) and the *final dormant phase* (*I*) are attained. Complete sterility of the culture may not ensue for weeks or months, depending on the kind of organism.<sup>3, 4, 5, 5a</sup>

**Factors Affecting Growth Phases.**—The form of the growth curve may be affected by many factors. For example, if the culture be suddenly plunged into icewater, the curve at once ceases its upward trend, remains flat for a time and then begins to decline. If held at 22° C. instead of 35° C. (for *E. coli*) the rise in the positive phases is much less abrupt and much more extended. Other factors such as *pH*, concentration of food, and so on have their effects also.

The state of the initial inoculum also affects the first part of the curve. If dormant cells are used, the curve is something like that seen in Figure 123. If cells from a culture in the logarithmic phase are used, and conditions of growth are equally favorable, the slower phases are nearly obliterated, a logarithmic phase beginning almost at once.

#### VARIATION OF BACTERIA

Variation of bacteria has long vexed the microbiologist and is one of the most fundamental and practical questions before him today. We must consider two doctrines in regard to this problem;

namely, *pleomorphism* and *monomorphism*. These terms are used in various senses. Strictly, pleomorphism means many forms and should apply to morphological features only. Since morphological and physiological characters commonly vary, however, the term has come to include the latter as well as the former and is used in that sense here. The more extreme among the pleomorphists hold that bacteria are fundamentally unstable, changing culturally, morphologically and chemically from one species, or even family or order, to another, going to ultramicroscopic, virus-like stages and passing through complicated developmental cycles analogous to those seen among higher plants and animals.<sup>6, 7</sup>

The extreme monomorphists hold, on the contrary, that all bacterial species are entirely stable; never changing in any respect; that there are no sexes and no multiplication except by fission.

Pleomorphism is the older view, and in its extreme form was the result, undoubtedly, of observations upon impure cultures made by numerous investigators prior to about 1875. It is easy to understand how, without the advantages of solid culture media, staining methods and good microscopes, these erroneous views could have prevailed.

About 1872, F. Cohn stated that he believed bacteria to be much less variable than had previously been thought. This was a great advance from extreme pleomorphism and accordingly aroused a storm of protest. When Koch studied bacteria a few years later he had the advantage of exact, pure culture methods and he kept transferring his cultures on the same sort of media under very uniform conditions so that they tended to remain uniform in character. He, better than anyone else at that time, could appreciate the deceptive role of contaminants in producing apparent changes in bacteria and, as a result, he arrived at the conclusion that the doctrine of pleomorphism was largely the result of contamination. There then arose, among his pupils, the doctrine of strict monomorphism. Much controversial literature has since been written by both schools.

The truth lies somewhere between strict monomorphism and extreme pleomorphism, and might be termed "limited pleomorphism," or *oligomorphism* which means, literally, "few forms." Probably no modern bacteriologist denies that bacteria vary and that they do so within fairly wide limits. If, however, we propound the question "to what extent, and why, do they vary?" we are answered by a chorus, not to say cacophony, of differing opinions.

There can be no question that variations in size and shape of cells, motility, capsules, pigment, and other characters may occur, often as a result of what seem to be very small irregularities in time and temperature of incubation, age of culture, composition of medium and other environmental factors. Some of the changes appear to occur spontaneously, *i.e.*, for no assignable reason. Similar variations, due to analogous causes, are seen in higher plants and animals.

Among the most striking and important phenomena of bacterial variation are those related to the form of the colonies on solid media. There is a voluminous literature on the subject, and dozens of different variant colony forms have been described.<sup>6, 7</sup> However, there are five or six familiar forms of colony variant which are most frequently seen and to which we shall confine our attention.



Fig. 125.—Colony variant of aerobic, spore-forming bacillus. Smooth or “S” type of colony ( $\times$  about 8).



Fig. 126.—Colony variant of same organism depicted in Figure 125. Rough or “R” type of colony ( $\times$  about 8).

**Rough and Smooth Colonies.**—Let us consider the colonies produced on infusion agar by a strain of a common, saprophytic, aerobic, spore-forming bacillus which was recently isolated from dust. The colonies ordinarily produced by the organism were about 2 mm. in diameter, gray and translucent, but appeared in two different forms. One form of colony was perfectly smooth, moist and homogeneous, convex, circular and glistening and had regular margins (Fig. 125). This is a well-known type of colony variant occurring in most species of bacteria and is spoken of as a *smooth* or “S” type of colony. The other type of colony was not glistening but rather dull, had rough or wrinkled surfaces, irregular edges, and was rather dry looking. This also is a well-known form of colony variant occurring in many species of bacteria and is called a *rough* or “R” type of colony (Fig. 126). It is commonly observed that the cells in rough colonies form themselves into long tangled

filaments on the surface of solid media (Fig. 132) whereas in smooth colonies the cells tend to occur singly. In broth the rough growth is



Fig. 127.—Colonies of a species of spore-forming aerobic bacillus showing sectors (light areas) of nonsporulating rods of the same species. (Photographs courtesy of Dr. J. Howard Brown.) ( $\times$  about 15.)

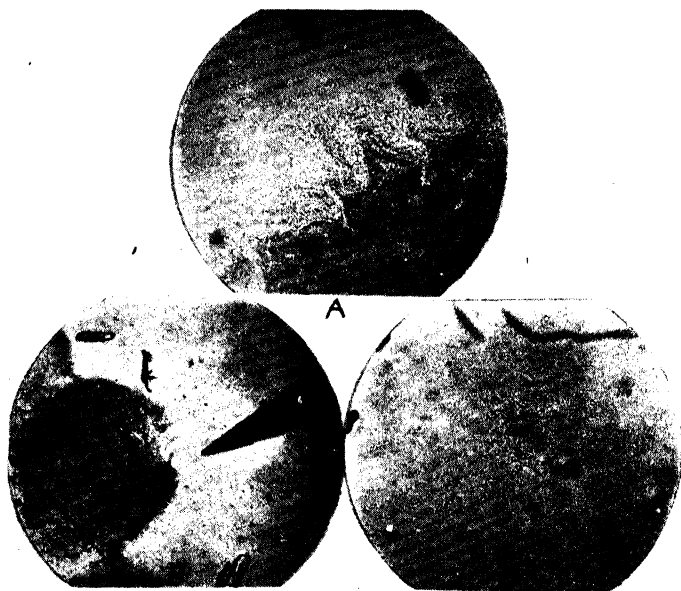


Fig. 128.—*A*, Margin of colony magnified about 500 times to show sporulating and nonsporulating growth. The dark, oval granules are spores, and the dark areas in the lower right part of *A* are masses of sporulating cells or filaments of cells. *B*, The tip of the micro-manipulator needle is seen teasing apart the margin of a colony so as to separate the sporulating from the nonsporulating growth. *C*, A very young colony developing from a single, asporogenous cell. This growth is only a few (4–5) hours old and is completely invisible except with the high-dry lens. This is growth of the *R* or rough type.

usually granular or flaky, the smooth growth producing an even turbidity. Practically all species of bacteria vary in this way.

The phenomenon of variation, especially as it relates to colony form, is often loosely spoken of as dissociation. However, the term "dissociation" is borrowed from the ionic theory in chemistry and

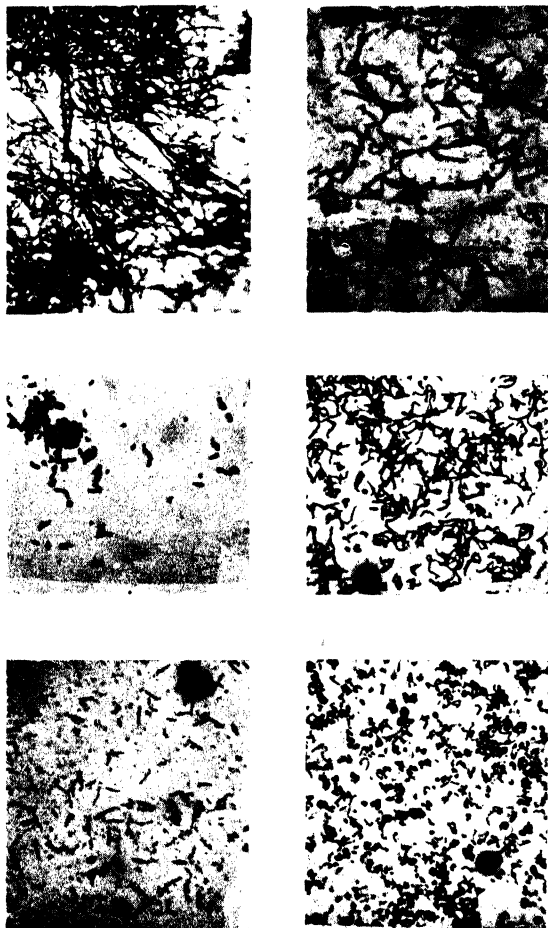


Fig. 129.—Different morphological types assumed by a single kind of organism ( $\times 900$ ).

should not be applied to bacterial phenomena. It implies the previous existence of the two types of variant in the culture. It has been shown, however, that the R and S forms can develop from the descendants of a *single cell* of either type.

When the R and S colonies of an organism are studied, it is generally found that the cells composing them have different properties, very striking departures from the "normal" (or most familiar) form sometimes being noted. Thus, in the spore-forming organism referred to above, colonies were frequently observed having sectors of nonsporulating cells (Fig. 127). By means of a very fine needle and an instrument called a micro-manipulator,<sup>7a</sup> these two types of cells (sporulating and nonsporulating) could be separated in pure culture (Fig. 128). It was found, on further study, that a single cell of either type could produce the other, *in either the R or S form*. No reason could be found to account for the variations. They *seemed* to occur spontaneously. Loss of spore formation is a common observation. In addition to this, cells of the organism being described, in different colonies often of the same

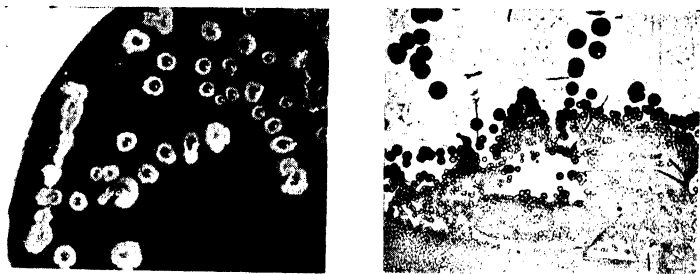


Fig. 130.—Colony variants of a species of spore-forming aerobic bacillus. Compare these colony forms with those seen in Figures 125, 126 and 131. (Natural size.)

type, varied so widely as to be unrecognizable as the same organism. Some of the morphological variants are shown in Figure 129.

Changes in the cell form of the same general nature are of common occurrence in many species of bacteria, but are not always exactly the same as these, often being much less extreme.

*The production of capsules* is usually associated with S type colonies, but not all S type organisms are *demonstrably* encapsulated. S type pneumococci have large, easily demonstrated capsules, but S type typhoid bacilli do not. Since capsules are frequently related to virulence, S types of pathogenic organisms are usually more virulent than R types. However, S forms of many harmless organisms are known, the spore-bearing bacillus referred to above being a case in point.

The chemical and antigenic composition of R and S variants

of most organisms differ, sometimes profoundly. This will be discussed more in detail in connection with specific organisms.

Since bacteria, like all living things, tend to adapt themselves to their environment and to overcome difficult situations, they often undergo marked variations when in contact with the resistance offered by disinfectants (see drug-fastness, page 97) and the animal body. On repeated transfer from one animal to another they often become more and more able to survive and grow in such a situation, and this is reflected in what is called an enhanced viru-



Fig. 131.—Petri plate showing mucoid or “M” type of growth of the same organism as is seen in Figures 125, 126 and 130. Natural size. At the right is seen an enlarged picture of the edge of one of the mucoid lobules. Compare with the edge of another type of colony seen in Figure 132. ( $\times$  about 15.) Both are organisms of the same genus.

lence. They often surround themselves with protective capsules and become smooth. Disinfectants alter them similarly.

On the other hand, if they are removed from the competitive environment, transferred repeatedly on ready-prepared media under the most favorable conditions, their virulence decreases, they often lose their capsules, become rough, and frequently become quite changed in various biochemical and structural details. They tend away from parasitism toward saprophytism. The R form usually reflects favorable conditions of growth, the



S form a reaction to unfavorable conditions, but there are many exceptions to this generalization.

**Mucoid Colonies.**—A strain of motile, spore-forming organisms similar to that discussed on page 247, when cultivated on infusion agar containing a little phenol, produced not only R and S types of colonies but ten or eleven other varieties wholly dissimilar from each other in appearance. Two of these are seen in Figure 130. Some of the colonies were viscous and slimy, like mucus. This kind of colony is common in many species of bacteria, both spore-forming and non-sporeforming, rods and cocci, and is called "*mucoid*" (Fig. 131). The mucoid matrix seems to be secreted by



Fig. 132.—Colony variant showing medusa head arrangement of filaments of bacilli at edge of colony. Compare with the mucoid colony seen in Figure 131. (Hagan, "The Infectious Diseases of Domestic Animals," Comstock Publishing Co., Inc.)

the cells in response to various external stimuli (in this case phenol) and may represent an attempt on the part of the organism to separate itself from an unfavorable environment.

Other colonies produced by the particular organism under discussion, when viewed with the microscope, were seen to be composed of flat masses of coiled, parallel strands of filaments arranged much as is a lady's hair when set in "waves." Colonies of such form are sometimes called "medusa head" or coiled colonies (Fig. 132). They represent the extreme development of roughness in the colony.

Pure cultures derived from these different colony forms varied

in spore formation and motility and in the *rate* at which they fermented different carbohydrates and digested different proteins. Observations upon these fermentative functions, made after 72 hours' incubation, for example, showed apparent wide differences in biochemical characters, but if the biochemical test cultures were all observed every day for 2 weeks it was found that all variants eventually showed the same physiological characters. Had observations been discontinued after 72 hours, the impression might have been gained that the original culture had split up into a number of new species having different physiological properties. This source of error is probably not an uncommon one. However, many organisms apparently undergo lasting and definite alterations in these respects, but care must be used in determining this fact.

**Minute or Small Colonies.**—Rough, smooth, and mucoid colonies are of common occurrence and are often relatively stable in character. Many other types, as has been said, may occur. The literature on the subject sounds somewhat alphabetical. There are R, S, and M types, O, H, X, and Y forms and others.

One rather unstable form, of a curious nature, is the minute or small colony. Some workers have suggested that these colonies represent gonidial (intracellular granule) developments, hence they are sometimes referred to as G colonies. It is impossible to illustrate these because they are so tiny as to be almost invisible. Many types of minute colony have been observed in cultures of diphtheria bacilli, and some are of common occurrence in staphylococci, dysentery bacilli, and numerous other species. The "G" organisms of diphtheria bacilli do not seem to differ in ordinary respects from the large colony types. These colony variants often tend to revert to the larger colony forms quite readily. Minute colonies may also be R, S or mucoid.

**H and O Variants.**—These are among the most interesting variant forms from the standpoint of diagnostic and systematic bacteriology. They were first described in connection with *Proteus vulgaris*, a common non-sporeforming, gram-negative, motile rod found in feces, stagnant water and decaying organic matter. When cultivated on agar plates or any solid medium, *P. vulgaris* usually spreads over the surface quite widely in a thin, smooth, grey, translucent film, by a process of extension called *swarming*. The individual cells in such growth are very motile, *i.e.*, they have flagella and swim out from the edge of the colony. This form of the organism has been designated the **Hauch** or H form (the German

word *Hauch* meaning a film or veil) to indicate the spreading, veil-like character of the growth. In many respects the H form of *Proteus* is closely akin to the S form of other bacteria. A second form of growth is observed when the colonies are small and discrete. In these the *Proteus* bacilli are found to be nonmotile, i.e., they possess no flagella. These are called the *Ohne Hauch* or O form (or "without spreading" growth).

The antigenic structures of these variants are of great interest, especially in relation to the diagnosis of typhus fever (see page 756), and also of typhoid fever and related diseases (see page 482). The phenomena are not isolated oddities but, like many other apparently curious and disconnected observations, are links in a broad and fundamental system of biological interrelationships, other parts of which are still hidden but which may be found in many species if investigation is made.

Motile and nonmotile variants of other organisms are common, and although the motile variants may not have the spreading "swarming" character (*Hauch* form) of *Proteus*, they are often referred to as H forms, meaning flagellate or motile, while the *Ohne hauch* forms are called O forms because they have no flagella. As will be seen later, the term H is often used to refer to the flagella themselves or to their proteins (H proteins) and the letter O to designate the body or somatic substances (O substances) of various bacteria.

**Secondary Colonies.**—The formation of small excrescences, papillae or outgrowths from ordinary colonies of many species of bacteria, after the first growth is mature and begins to age, is a curious but common phenomenon. The outgrowths are called secondary colonies or "daughter" colonies. They may appear on the surface, develop from within, or grow out from the edges (Fig. 133). They vary in size, form, numbers and appearance. Usually the cells in secondary colonies vary from the original in many properties, both morphological and physiological.<sup>8</sup>

The reason for their formation is not clear. Apparently, after the original colony matures, conditions for further normal growth become unfavorable and the cells become unstable, varying in response to the changed conditions. The secondary growths may be of R, S, or mucoid types; and transfers from them may produce cultures with altered antigenic structure, fermentative properties, etc. Such variants of *Escherichia coli* are especially well known. One variety of this species, formerly referred to as *E. coli mutabile*, was so named because of its tendency to produce secondary

growths with fermentative properties differing from the parent growth.

**Other Variants.**—It is impossible in this brief space to describe all observed bacterial variants, but it may be said in passing that colonies of pigmented organisms vary in color, motile species give off nonmotile variants, virulent ones become nonvirulent, and so on.<sup>9, 10, 11, 12, 13, 14</sup>

**Variations in degree** may also occur. Thus, an organism producing much toxin may gradually produce less and less as successive

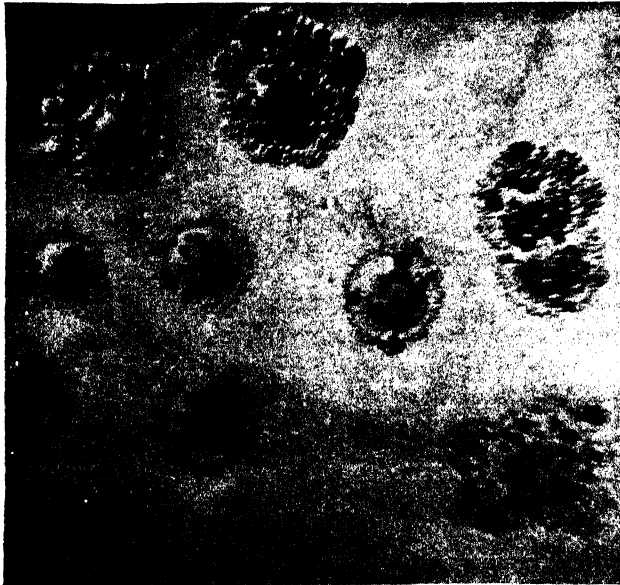


Fig. 133.—Colonies of *Eberthella typhi* (Dorset Strain) showing secondary or daughter colonies. Magnification  $\times 10$ . (Army Medical School.)

cultures are made, yet never lose the property of toxigenicity entirely. A potent lactose fermenter may become a weak fermenter and so on. Different strains of the mold *Penicillium notatum* vary greatly in penicillin production.

**Independence of Characters Which Vary.**—In any organism, variants of either the R or S or some other type may, when cultivated on the proper media, produce colonies of any other sort, with cells having various and unpredictable combinations of properties. Sporulation, in those species in which it occurs, is not

necessarily related to either R or S type. Combinations of any characters may appear in the variants of a given organism. If, for example, the organism happens to be virulent for certain animals, the virulence may be associated with the S type or some other type. Pigment formation and virulence may go together in a given organism, but are not necessarily always present together in that organism. One of the organisms described in a preceding paragraph (page 247) was observed to form nonsporulating colonies which were very large and of a *dark brown color*.

It is difficult to predict what characters may be associated. Mendelian laws do not *seem* to hold in this sexless (?), chromosome-free (?) world of bacteria. This is quite different from the regular association of certain characters which takes place in higher plants and animals. Thus, hemophilia or "bleeding" which occurs so tragically in some of the royal families of Europe, is linked with sex.

**Mutation.**—The appearance of *permanent* variants, analogous to "sports" in higher plants and animals, is claimed by many to be of common occurrence in bacteria. On this basis the belief is held by some that many of the present so-called "species" are merely permanent variants or "sports" of some central type. The question is unsettled. *It is characteristic of most variant strains of bacteria, however, that they tend to revert eventually to the parent (i.e., most familiar under the existing conditions) type.*

**Possible Cause of Variation.**—Just *why* bacteria vary is unknown. One explanation already mentioned, assumes that the variations represent stages in life cycles.<sup>6, 7</sup> Another is based on the hypothesis that the changes are in the nature of hereditary mutations involving chromosomes and sexes but since, as we have shown, there is little confirmed evidence for the existence of these in bacteria, such an explanation would seem inadequate at present. Most of the variations seem to occur in response to environmental conditions. But of the *mechanism* of the variations we have only vague notions.

It would appear, after consideration of the data available, that in bacterial variation we have to deal with a rather loose arrangement of primitive, character-bearing bodies which are not organized to the same extent as chromosomes, or even as genes. Probably they are merely grains of chromatin-like material scattered throughout the bacterial cell in very unstable arrangements. These arrangements, presumably, are easily disturbed by the action of various external stimuli, so that when the bacterial cells divide, irregular combinations or divisions of the character-bearing bodies occur,

resulting in variations of the bacteria. The relationships may be imagined as analogous to the number combinations that result from rearrangements of a group of dice.<sup>15, 16, 16a</sup>

Sometimes the effects of the stimuli affecting the character-bearing bodies may be chemical or irreversible, the result being what might be termed permanent "sports" or *genetically stable variants*. Such variants continue unchanged through many generations. Their occurrence is believed to be rare in bacteria.

The reversibility of changes such as occurred in the organisms described above suggests that in these the character-bearing bodies were affected in a physical rather than in a chemical way, since the variants tended to revert to the original forms. The different forms of these organisms might be designated as *genetically unstable variants*.

The possible role of radiations of various sorts—x-rays, radium radiations, etc.—in inducing genetically stable changes in bacteria as they do when the gonads of higher organisms like fruit flies are exposed to their influence has been investigated by Spencer, and the field should be further explored.<sup>17, 18</sup>

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## CHAPTER 13

### MICROBIAL ASSOCIATIONS

IN experimental studies of bacteria, the greatest strides have been made since the development of reliable methods for obtaining pure cultures. Prior to Koch's time a great deal of confusion had existed in bacteriology because so much work was done with cultures which, often unknown to the investigator, contained more than one kind of organism. One result of this was the development of a great many extreme views on pleomorphism of bacteria and a perplexing literature on the subject. The importance of pure culture methods, in bringing at least a modicum of order out of this chaos, cannot be overstated. Indeed, for many years pure culture study has almost entirely superseded mixed culture study for most purposes. In some respects, however, it is perhaps unfortunate that this is so largely the case. In our desire to learn about individual kinds of bacteria *per se* we have overlooked their environment, their interrelationships and their mutual interactions in various commercial processes and in disease.

In outlining a study of man, we could not consider the program complete which did not include a review of social and political relations, including the widest international relations. With creatures as large and traceable and well understood as human beings, interrelationships between individuals, and groups of individuals, and their environments, are more or less readily studied and are seen to be of the utmost importance. So also, with other creatures visible to the naked eye; interdependences, symbioses, antagonisms, parasitisms and the like relations are part of life and, when living creatures are removed from such natural environmental influences, they undergo change and when so studied give only a distorted image of life.

Any student of nature has encountered many examples of biologic interrelationships. A simple type of antagonism or incompatibility is seen between trees and grass. Where the ground is constantly shaded and the moisture taken by the trees, grass is excluded. Cats and dogs usually furnish a striking example of incompatibility. A great many examples of symbiotic or mutually advantageous relationships might also be cited. The lichen, which consists of a fungus and a green alga, is a popular illustration. The fungus protects the alga while the latter furnishes certain food substances in a state assimilable by the former. An extremely interesting example is found in the relation of bacteria, protozoa and termites. Termites live on wood. The termite, however, is quite incapable of digesting wood. This function is performed by protozoa and bacteria inhabiting the gut of the insect. The termites are absolutely dependent on the life of the protozoa since, if the latter are killed by certain procedures, the termites die, full of undigested wood. The protozoa, in turn, thrive only in the gut of the termite. Ants which cultivate, protect and feed plant-lice receive, in return, the honey-like secretion of the lice and a mutually beneficial relationship thus exists. So also man feeds and protects cattle, which in turn furnish him with food, clothing, fertilizer and many other valuable articles.

All of these examples illustrate various types of interrelationship and interaction which arise from the struggle for existence, and which we are forced to study when we investigate nature in its macroscopic forms. Is there any good reason to suppose that microscopic creatures like bacteria are exempt from the necessity to struggle for existence? Many of the conditions constituting their environment are created by the growth of other bacteria. If we eliminate these conditions, as is generally done in pure



culture studies, we obtain a curiously unbalanced knowledge of bacteria.

Consider, for example, some minute recess a few inches below the surface of a soil rich in organic matter and moisture. Here we may find strict aerobes and anaerobes growing, decomposing cellulose and protein substances into simpler compounds which are available to nearly all the soil bacteria. These and other forms produce compounds of an endless variety, all available to the soil flora and fauna. The growth of the anaerobes may be favored by the utilization of excess oxygen, or catalase production by the aerobic bacteria. Here also the fertility of the soil may be increased because autotrophs grow, forming nitrites from ammonia produced from proteins by the proteolytic heterotrophs; and nitrobacter producing nitrates from the nitrites. The symbiotic relationship between leguminous plants and the genus *Rhizobium* is elsewhere discussed in detail (see section on nitrogen fixation, page 410). At the same time, all of these bacteria may be held in check and perhaps killed by the poisonous substances poured forth by certain of the Actinomyces, molds and *Pseudomonas* group, spore-forming bacteria alone outliving all others. The constant necessity for adjustment to changing temperatures and changing osmotic pressures, as well as changing composition of suspending fluid due to fluctuation of water—conditions which are fairly constant in most artificial media—also probably affect the bacteria. In another section of this book a more detailed discussion is given of certain substances given off by species of *Penicillium*, *Actinomyces*, and *Bacillus*, which are of such highly antagonistic nature that they have assumed an enormous importance in the treatment and control of infectious disease (see penicillin, gramicidin, pages 132 and 140).

The growth of many of the autotrophs in the presence of much organic matter seems incompatible with observations made in test tube experiments, yet in their natural environment, as in manured soils, they are constantly in the presence of organic matter. Do other bacteria produce changes favorable to their growth? It seems highly probable, but no one knows.

It is plain, therefore, that rapidly changing environmental vicissitudes and interactions between various forms of life are the usual experiences of free-living bacteria, as contrasted with growth in isolated tubes and completely segregated communities under the most absolutely uniform and favorable conditions possible in the laboratory.

Even those strict parasites which never survive in nature outside

the body of their host seldom find the site of their entrance into, or growth upon, the body devoid of other bacteria. The science of immunology has taught us something about reactions between pathogenic bacteria and their animal hosts, but our knowledge of the nature and effect of the saprophytic or potentially pathogenic bacteria often present at sites of infection, upon invading bacteria is relatively slight.

Our ignorance of bacterial interactions is not, however, of an absolutely Stygian hue. Pasteur enlightened us with his observation of the respiratory relation between aerobes and anaerobes. Some later experiments have shed light also. For example, it is now an old observation that on heated blood agar plates streaked with swabs from the throat, *Hemophilus influenzae* colonies grow best in the immediate neighborhood of colonies of *Staphylococcus aureus*. Evidently *Staph. aureus* produces some substance favoring the development of *H. influenzae*. Eggerth found, like others, that *H. influenzae* would not grow in plain broth devoid of blood. Yet *H. influenzae* grew very well when, in this blood-free broth, there was immersed a collodion sac containing a growing culture of *Staph. aureus*. Something which we now know to be coenzyme (see section on genus *Hemophilus*, page 631) passed through the sac from the staphylococci to the *H. influenzae*. Streptococci and pneumococci were likewise found to produce some growth-promoting substance which diffused, from the pure culture inside the sac, to the pure culture of *H. influenzae* in the broth outside surrounding the sac. This was a definitely favorable interrelationship, although benefiting only one of the species involved. Such a one-sided relationship is sometimes called *commensalism*. When both members of the partnership benefit, the relationship is called *symbiosis*.

**Antibiosis.**—Frost<sup>1</sup> demonstrated a reverse action. He immersed collodion sacs, containing pure cultures of *Eberthella typhosa*, in cultures of various soil bacteria. *Bacillus vulgaris*, *Pseudomonas vulgaris*, *Ps. fluorescens* and others had a definitely antagonistic action on *E. typhosa* even though separated from it by a collodion membrane. The antagonistic substances did not exist ready-formed in soil or water, but were formed only by the rapid multiplication of the antibionts in the immediate presence of *E. typhosa*. Several species of bacillus, as *B. subtilis*, as well as other organisms, produce substances lytic for parts of organisms (substances related to gramicidin dissolve the capsules of pneumococci) or whole cells. Cultures of *Leptospira icterohemorrhagiae* are killed by growth of many common contaminants in the laboratory. The term *antibiosis* or

bacterial antagonism is sometimes applied to such unfavorable relationships.

*Pseudomonas aeruginosa* also exemplifies the very important relationship of *antagonism* among bacteria. If a flask of broth be inoculated with a pure culture of some such organism as *Staphylococcus aureus* or *Eberthella typhosa*, millions of the organisms will have developed in each cubic centimeter of the broth after 24 hours' incubation at 37° C. If the culture now be inoculated with *Ps. aeruginosa*, an examination after some hours of incubation will show that the multitudes of *Staph. aureus* or of *E. typhosa* have been greatly reduced in numbers or entirely destroyed. Certain growth products of *Ps. aeruginosa* are highly poisonous for a variety of other organisms and, in mixed cultures, permit the predominance of *Ps. aeruginosa* over many other bacteria.

**Synergism.**—The term *synergism* is used in pharmacology to express the combined action of two or more drugs. As Holman has pointed out, in theology it signifies "the combined action of the human being and the divine grace in the salvation of the soul."<sup>6</sup> The term *bacterial synergism* is used to express the production of reactions by two or more species of bacteria growing together, which are not produced by either of the organisms when growing alone.

A number of very interesting examples of bacterial synergism have been brought to light, most of which fall into one of two general types. One of these two types is illustrated by the phenomenon described by Burri and Stutzer in 1894.<sup>2</sup> These workers showed that *Escherichia coli* and *Bacillus denitrificans* produce nitrogen from sodium nitrate. Neither alone can do this. The explanation is relatively simple. *Esch. coli* reduces nitrate to nitrite but no lower. *B. denitrificans* can reduce nitrite but not nitrate to nitrogen. If a nitrate-broth tube, in which *Esch. coli* has been growing, is sterilized by heat or filtration and then inoculated with *B. denitrificans*, the nitrite remaining from the metabolism of *Esch. coli* is reduced by *B. denitrificans* to nitrogen. *Simultaneous growth is not necessary.* This is the important point in this type of reaction.

The second type of reaction, originally described by Nencki in 1892,<sup>3</sup> has been extensively studied but remains much more obscure than the type just described. It is illustrated by the following, later discoveries.<sup>7, 8, 9</sup>

If cultures of *Streptococcus faecalis* and *Escherichia coli*, neither of which produces gas from saccharose alone, are mixed under certain

conditions, gas will be formed from saccharose by the combined growth. A number of such gas-forming pairs have been described by different authors. Such combinations as *Streptococcus faecalis* and *Salmonella paratyphi* or *Proteus vulgaris* (in saccharose and lactose), *Eberthella typhosa* and *Proteus vulgaris* (in mannite), *Streptococcus faecalis* and *Klebsiella pneumoniae* (in lactose), *Staphylococcus aureus* and *Salmonella icteroides* (in saccharose), *Streptococcus viridans* and *Proteus vulgaris* (in mannite) and *Shigella dysenteriae* and *Proteus vulgaris* (in mannite) all produce gas from the carbohydrate named, which yields gas to neither organism alone of any pair. Occasionally a false positive presumptive reaction in sanitary water analysis is obtained by such gas-forming pairs (see section on examination of water, page 432).

It will be observed that, in each pair, one organism is capable of fermenting the given carbohydrate with *acid formation*, while the other ferments *dextrose with gas formation*. The most obvious explanation is that dextrose is probably formed through hydrolysis of the higher carbohydrate by the acids resulting from its fermentation by one of the pair. The reaction differs from that of the Burri and Stutzer type in that a sterilized culture (heated or filtered) of one of the pair will not yield gas when inoculated with the other. Only through the *intimate contact* and *simultaneous growth* of young, active cells in *certain proportions* and of *certain ages* is the phenomenon made manifest.

These test-tube studies illustrate bacterial synergism in its simplest form. We have at least some control over the processes and can observe and repeat and vary them at will. Much more complicated and difficult to analyze are certain interactions between pathogenic bacteria and the infected host. Examples of such complicated interaction are found in the production of gas gangrene by such organisms as *Clostridium perfringens* and related clostridia; *Corynebacterium diphtheriae*, when infecting the throat, is never free from the effects of the normal bacteria of the mouth; streptococci, when causing scarlet fever or septic sore throat, also encounter other bacteria; *Eberthella typhosa*, in the intestine, is surrounded by numerous species and myriads of cells of other bacteria.

An attempt to analyze at least certain phases of the host-parasite relationship was made in 1893 by Vincent.<sup>4</sup> This author presented a very careful piece of work on the interaction between *Eberthella typhosa*, streptococci and the rabbit. Vincent observed that streptococcus sore throats or skin infections in typhoid fever

are usually associated with severe and fatal cases of the disease. even though the streptococcus infection may not occur till after fever has disappeared. He gained the impression that the typhoid bacilli "préparent un terrain éminemment favorable" for secondary invaders, especially streptococci. The investigator could inoculate either organism alone, intravenously or intraperitoneally, into rabbits or guinea pigs (naturally resistant to infection with typhoid bacilli) without producing severe infection. Mixtures of the two organisms, however, killed nearly all the rabbits within two days or longer. For example, a rabbit was injected intravenously with *E. typhosa*. Streptococci were then applied on a compress over a scraped area of skin. The rabbit died in 8 days and cultures showed both organisms in all organs. If the injections were made separately or at distinctly different times, no untoward result occurred. The two had to be together. This suggests the Nencki type of synergistic reaction. Further experiments showed that the streptococci favored invasion of the body by *E. typhosa* through the ability of the former to check the action of the leukocytes against the latter.

The Vincent type of reaction, in which the resistance of the host is lowered or held in abeyance by one organism while one or both invade, is probably common enough in many pathological conditions. For example, the virus of swine influenza, alone, causes a relatively mild epizootic disease. When there is superimposed upon this disease an infection with a certain organism of the genus *Hemophilus*, the epizootic\* frequently becomes a fatal scourge.

A striking example of pathogenic synergism was observed by Meleney<sup>5</sup> in a man recovering from an operation for appendicitis. In the skin and subcutaneous tissues closely surrounding the drain and stitches there sprang up an inflammatory reaction which spread outward rapidly, the center portions becoming gangrenous. Excision of the gangrenous portion had no effect. It was necessary to excise all the superficial tissues over an area extending considerably beyond the outer edges of the inflamed tissues.

Observation of the extreme, marginal portions of the inflamed skin showed streptococci in the tissues. These were present in pure culture. In the inner, gangrenous zone there was present, also, a staphylococcus. Apparently the streptococcus invaded the tissues ahead of the staphylococcus, producing inflammation; the latter organism followed and produced, in combination with the

\* An epizootic is, among animals, the analogue of an epidemic among human beings, that is, an unusual prevalence of some infectious disease.

streptococcus, the gangrene described. Neither, in pure culture, caused gangrene when injected subcutaneously into animals (dogs) nor when injected in sites a little way apart on the same animal. Only when *mixtures* of the two live organisms together were injected did gangrene develop, and then it was extensive.

The explanations of most of the various bacterial interactions described in this chapter are obscure or entirely wanting. So little is known of the enzymes given off by bacteria, not only in a culture tube but in response to the resistance of a host, or complex environmental conditions such as exist in the intestine or in the soil, that no really accurate description of these synergistic reactions is possible. We do know, however, that bacteria seldom live alone, that the usual pure, artificial cultures give but a partial idea of bacterial processes and that, in the field of bacterial synergism, the clever and curious investigator will find enough problems to claim his interest and tax his utmost resources for many years to come.

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## CHAPTER 14

### BACTERIA AND DISEASE

WE HAVE already seen that many microorganisms have become adapted to growth in or upon the tissues of plants and animals (insects are also subject to some microbial infections) and that by such growth they disturb the normal functions of the infected hosts, sometimes to an inappreciable extent, sometimes severely, and sometimes fatally. Obviously the development of disease, its severity and its nature, will depend on the kind of reaction that occurs between the host and the infecting organism, as well as on the species of host and of microorganism involved. In later chapters disease-producing agents which are neither bacteria nor protozoa, *i.e.*, viruses and rickettsiae, will be discussed. Here we shall deal with bacteria in their pathological relationships.

Except for a dozen or so genera, bacteria are quite harmless and many are, indeed, entirely indispensable to the continued existence of higher forms of life on the globe. With those bacteria that are engaged in maintaining the fertility of the soil and those which act as scavengers we have, in our everyday life, little concern and we can in no way control them except in the most limited sense. Certain species are of industrial use or are important as causes of decay, spoilage, rot and fermentation, and we therefore either exploit them and induce them to serve us or devise means of killing and avoiding them. With respect to those few species which have taken on the habit of parasitism and disease production, man has for many years been engaged in the most intensive study and has expended incalculable amounts of money, time, energy and human and animal life in efforts to combat or exterminate them. Veterinary and human medical studies have unearthed many fundamental principles of microbiology, many of them directly applicable in agriculture and the manufacturing arts, but because of the importance of, and interest in, the few pathogenic species, several phases of medical bacteriology are discussed in considerable detail in this book. However, it is of interest to remember that one of the foremost founders of bacteriology and immunology was primarily an industrial microbiologist who did much of his fundamental research in this field. The name of this man is well known—Pasteur. Many of his greatest discoveries were made also in the field of veterinary science.

**What Is Disease?**—In order to understand disease, we should know what it is and why and how it occurs.

An exact definition of disease is difficult of formulation. We may say that disease is a departure from normal. But what is normal, and how great must be the departure before it falls into the category of disease? We may say that disease is present when injury results in visible or sensible departures from that condition which enables the host to function in a manner most advantageous to itself. But how are we to know when a host is functioning to its own best advantage? Possibly the simplest plan for the present is to fall back on the generally accepted meaning of the term disease and leave such academic arguments to the lexicographers. Disease, we shall say, is any visible or sensible, *harmful* departure from the "normal" condition of the body. Such conditions often result from the entrance into the body of organisms which are able to multiply in, *and to damage*, body tissues. The entrance of such organisms into the body is called *infection*. Disease-producing agents are called *pathogenic* (*pathos* = sadness; *genic* = producing).

Disease may also result from the entrance into the body, *by certain definite routes*, of poisonous metabolic products of micro-organisms. Examples of these are diphtheria toxin and botulinus toxin.

**Parasitism and Pathogenicity.**—A distinction must be drawn between *parasitism* and *pathogenicity*. A wholly effective (but hypothetical) parasite is one which is wholly nonpathogenic and yields absolutely no benefit to its host. A differentiation between this relationship and commensalism might prove difficult. Such a parasite does no damage, stirs up no reaction against itself and so is left undisturbed to live out its ungrateful existence. Most *actual parasites* (as distinguished from commensals), however, occasion *some* damage to their host, although perhaps little. Some do so much damage that they soon kill the source of their livelihood and in this respect are inept parasites—possibly beginners in the evolutionary scale of parasitism. These are effective *pathogens* but not highly adapted *parasites*. As an analogy we might consider a skillful, time-tried and prudent burglar (parasite) who does all he can to avoid waking the resentment of the sleeping householder (host) whom he is about to rob. If he causes too much disturbance he may be cast out by the host, or he may kill the host and be deprived of the benefits of his parasitism and lose his own life as well. Thus we see that parasitism involves various degrees of pathogenicity.

On the other hand, pathogenicity does not always involve para-



sitism. For example, certain of the gas gangrene organisms and the tetanus organism cannot invade normal tissues, but live only in dead, necrotic material, causing disease by mechanical means (gas pressure) or by poisonous excretory products. The organism of food poisoning, *Cl. botulinum*, likewise cannot multiply in the body (with the doubtful exception of the intestine where it is not known to do harm) but produces a poison in foods outside the body which becomes effective when swallowed.

**Pathogenicity Is Fortuitous.**—Those not familiar with microbial disease sometimes conceive of bacteria (we shall use the term *bacteria* here to include all microorganisms) as vicious, spiteful creatures which have purposely set themselves to plague higher creatures with disease, producing poisons and invading the tissues for the sole purpose of causing harm. They are sometimes thought of as being endowed with special powers for injury. This is entirely wrong. We have just seen that excessive injury to the host will also result unfavorably to a parasite. The aim is toward commensalism not cannibalism! Damage to the host is purely accidental and not necessary to the parasite. As some trees in warm climates furnish good pabulum, freedom from competition with soil-growing plants, and other especially favorable conditions for growth and multiplication of parasitic plants like mistletoe, so a man, animal, plant or insect may serve certain bacteria as a good source of food, easy living, protection from enemies, and avoidance of competition. These are four of the main considerations in the struggle for existence. Most soil-inhabiting bacteria lack the physiological properties which might enable them to multiply within a host. If we consider man as the host, many bacteria of the soil find the temperature too high. Even though the temperature be suitable many cannot live in contact with organic material. Others require cellulose as a source of carbon. Still others require a different pH, and so on. Some find conditions in or on the body which will permit their growth but they cannot resist the action of the leukocytes. The first requisite to successful parasitism, then, is ability to live in or on the host without stirring up a reaction with which the parasite cannot cope. This ability seems to have developed as a process of evolution. The few that could adapt themselves did so.

*The process of adaptation* between host and parasite must be thought of as a mutual one, requiring many generations of natural selection of the most adaptable. Bacterial generations are very short, a few minutes or hours; human generations are long, about 25 years. Thus bacteria become adapted to growth in contact with

human tissues before man becomes tolerant to invasive bacteria. We may imagine that a few bacteria are just beginning the process of adaptation as they stir up a violent reaction (acute disease) every time they come into contact with the host and begin multiplying. Gonococci and pertussis bacilli are examples. The host, on the other hand, has not yet become adapted to them. Others are better adapted; the host, through many generations of contact, seems to have become more used to having them growing within him, and chronic disease ensues. Syphilis, tuberculosis and leprosy may be thought of as examples. In still other instances adaptation may be complete and might be represented by the apparent commensal relationship between the harmless organisms of the throat, skin and intestine, and the host.

In the process of adaptation both host and parasite become altered. This is implied in the word adaptation. These changes represent attempts by each to live comfortably under the new circumstances, and are not necessarily the result of a combat. However, the term *invasive* is often applied to bacteria as though they were imbued with the lust to conquer. Likewise the term *resistance* and *defensive mechanisms* are often applied to host factors which are unfavorable to multiplication of the parasite. We shall use these terms for convenience but their true significance must be constantly borne in mind.

As we have just seen, most bacteria have a very hard time of it when they come into contact with the human body. However, under some circumstances, certain kinds of bacteria do gain entrance to the tissues, often producing disease. The question then arises as to how these bacteria get in and how and why they produce disease, while others do not.

**Portal of Entry.**—If the skin be kept intact, no ordinary bacteria can get through it. But if any slight cut or scratch exists, then bacteria can get into the tissues. The thin membranes about the eye (conjunctivae), in the nose and throat and in the genitalia are less able to withstand bacterial invasion than the tough outer skin, and infections frequently begin in such situations.

The portal by which an organism enters the body is an important factor in determining the occurrence and kind of disease. Certain bacteria can establish themselves in the body only by first coming into contact with the respiratory tract, others only through contact with the intestinal lining and so on. Thus, dysentery bacilli (*Shigella dysenteriae*), rubbed over the hands, would cause no infection while, if swallowed, they might produce a fatal disease. On the other

hand, *Neisseria gonorrhoeae* might be swallowed without harmful effect but if rubbed in the eye or genitalia would cause gonorrheal infection of the mucous membrane.

The skin and mucous membranes of the body are inhabited by large numbers of bacteria, some of them highly pathogenic under some circumstances. They are usually held in check by phagocytic tissue cells, the skin and leukocytes. The secretions of the body are also important defenses against these bacteria. Some of the body fluids, such as perspiration, gastric juice and the vaginal secretion, are so acid as to destroy many bacteria. Even though not killed by the acidity, they may be removed mechanically by the secretions. Thus, bacteria caught on the sticky surfaces inside the mouth, nose or lungs are moved upward by the cilia of the epithelial cells lining the air passages

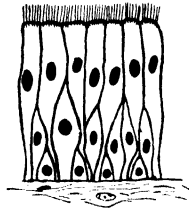


Fig. 134.—Section through the wall of a bronchus, showing the ciliated epithelial cells. These cilia are in constant motion carrying dirt and bacteria upward toward the mouth. The walls of the trachea and nasal sinuses are similarly lined with ciliated epithelium. (Burdon, "Medical Microbiology," by permission of The Macmillan Co.)

and then removed in the mucus and saliva by coughing, swallowing or sneezing (Fig. 134). Leukocytes are found in the secretions and are often seen in gram-stained smears of nasal or vaginal mucus, or saliva, sometimes with bacteria which they have ingested.

**Factors Favorable to Bacteria.**—Once gaining access to the body, ordinary saprophytic bacteria are usually immediately ingested and destroyed by leukocytes. Pathogenic bacteria, however, frequently have the power to excrete enzyme-like substances which kill and drive away leukocytes. Such substances are called *leukocidins* and *anti-opsonins*, respectively. If the invaders avoid or destroy the leukocytes and certain other tissue cells which phagocytize, they may have an opportunity to grow in the body and they may or may not excrete poisonous waste substances called *toxins*. The latter kill or injure the tissue cells and may paralyze

the defenses of the host and enable the bacteria to multiply further, using the body substances of the host for food. As the bacteria grow, they often undergo variations, becoming more adapted to the host, and better able to multiply within it. For example, they may acquire capsules which protect them. They may then proceed to grow into the lymph spaces, spreading widely through the tissues. Some, however, the diphtheria organism for instance, remain entirely localized. Others may grow in the blood stream and then we have the condition called *septicemia* or "*blood poisoning*." Having gained entrance by a favorable portal (*i.e.*, a tissue favorable to their growth or maintenance), they may be carried by the blood and localize at various other points in the body, *e.g.*, liver, spleen, bone marrow or lymph glands of the intestine, causing what are called *secondary abscesses* or *secondary foci of infection*, or *metastatic infections*.

**Toxins.**—The toxins of bacteria are of two sorts: *exotoxins* and *endotoxins*. Exotoxins are those poisonous waste products which are excreted by the bacteria into their environment. They are not to be thought of as a skillfully devised weapon directed at the host. It just so happens that they are poisonous. It would perhaps cause less embarrassment to the parasite if the excretions were more bland. They are produced anywhere that the bacteria grow, in a culture tube or in the body. Endotoxins remain within the bacterial cell. Some endotoxins may actually be the cell protoplasm itself. Most bacterial toxins are proteins or are substances associated with proteins. Some are carbohydrates as in pneumococci and type B influenza bacilli. Just how they act in the body is not clear. Some, as the toxins of *Clostridium botulinum* and certain staphylococci, are harmful when swallowed. Other toxins, like that of diphtheria bacilli, can be taken by mouth with impunity, but if injected or absorbed into the blood in very tiny doses may cause death. Many of these bacterial toxins are far more potent than cobra venom, but are produced in very minute amounts.

**Virulence of Bacteria.**—The ability of the bacteria to invade and grow in the body tissues and injure the body is spoken of as *virulence*. Virulence may conveniently (but not very accurately) be regarded as made up of two factors: *toxigenicity* (or ability to form poison) and *aggressiveness* (or ability to grow in the tissues and spread through them).

Virulence ↗ toxigenicity.  
                  ↘ aggressiveness (or vegetative power when in the body).

Obviously, each of these factors is dependent upon, and the result of, other properties, and the effect upon the host is dependent upon a balance between his own resistance, adaptability, and the virulence of the bacteria.

**Host Defenses.**—We have already mentioned several factors in the animal host which, being unfavorable to the growth of bacteria, are cited as defensive mechanisms. We have also pointed out that when bacteria find conditions of infection under which they can persist, the host attempts to adapt himself to the parasite. Many of the changes which occur in the host are entirely favorable to him and without much effect on the parasite. For example, a diphtheria patient may become able to neutralize the toxin of the diphtheria bacillus by producing antitoxin, yet the bacilli may continue to live very satisfactorily in his throat. The patient becomes a “carrier.”

**Carriers.**—Sometimes persons who recover from mild or severe attacks of contagious bacterial disease remain immune although still carrying the infective agent about with them. Such persons may spread the microorganisms abroad for days, weeks or years. They are called “carriers,” and it is largely by them that certain diseases are disseminated. Often carriers of typhoid, meningitis, pneumonia and other organisms occur without any *visible* disease or process of adaptation. We shall have occasion to mention carriers in discussing infectious diseases, so that the student should fully understand their importance at this point.

Other processes of adaptation of the host are unfavorable to the bacteria and then the latter may be killed. In such processes certain tissues and the leukocytes become more adept at phagocytizing the bacteria. Also, certain tissues produce substances that dissolve or clump the bacteria. These substances (along with antitoxin) are included in the term *antibodies* and their study constitutes, in part, the science of immunology which will be discussed in the next few chapters.

**Koch's Postulates.**—It is not always possible to be certain that bacteria which the bacteriologist isolates from a given disease lesion or from pus, blood or feces are the *cause* of the observed disease condition. Many harmless bacteria are found growing in feces, sputum and ulcerating wounds, and some would not grow there unless some other diseased condition existed first. To *prove* that bacteria are the *cause* of a given disease requires the most painstaking and careful study.

The question as to the *etiological relationship* of bacteria to any

disease was a very live one long before the time of Koch and there was much loose discussion and profitless argument regarding many bacteria and their relation to disease. When Koch established the pure-culture technic it became possible to apply exact methods to the study of the etiology of disease. He was very conservative in stating the relationships of any given germ to any particular disease.

His ideas on the subject were crystallized largely by his studies of the relationship of *tubercle bacilli* to *tuberculosis*. Koch, like others before him, observed the bacilli in the lesions of animals dead of the disease. But he was not too ready to believe that he had discovered the cause of tuberculosis because he found the organisms present in the lesions of tuberculosis. Might not this bacillus appear in the tissues merely accidentally because the animal, being so ill, is too weak to resist its invasion? Might it not be merely a relatively harmless opportunist? Might it not represent contamination with a common saprophyte? Koch, involved in a discussion of the problem, finally stated what he believed to be the evidence necessary to prove an organism to be the cause of a disease. The evidence consists of four postulates, generally called *Koch's postulates* today, and they are, essentially, as follows:

1. The organism must be associated with all cases of a given disease and in reasonable pathological relationship to the disease and its symptoms and lesions.

2. It must be isolated from victims of the disease in pure culture.

3. When the pure culture is inoculated into susceptible animals or man, it must reproduce the disease. Many such inoculations into man have been made on courageous volunteers. In others, accidental infections have occurred which have provided long-wanted evidence. The value of animal experimentation is here very evident.

4. It must be isolated in pure culture from such experimental infections.

It will be found even today that the etiological relationship of some bacteria to diseases which they are generally thought to cause has not been established firmly on the basis of Koch's postulates. However, circumstantial evidence is usually available in such cases.

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complished in several ways. For convenience we may list these as follows:

1. Mechanical, by:

- (a) objects (fomites\*)
- (b) droplets of saliva or nasal secretion
- (c) dust infected from any source
- (d) feces, and materials contaminated therewith, such as drinking water, milk and other foods. These are discussed elsewhere (see pages 476, 783, 795)
- (e) direct contact, as in the venereal diseases

2. Biological, by:

- (a) biting insects
- (b) the feces of insects

Transmission by water and sewage is discussed elsewhere (page 431).

Many diseases may be transmitted by several of these methods; others are restricted, under natural conditions, to only one or two.

**Mechanical Transmission by Various Objects.** (*Fomites.*)—It is obvious that any object having live, infectious bacteria upon it may, under certain circumstances, serve to transfer the bacteria from one person or place to another, causing transmission of disease. Thus, soiled bed linen or clothing, towels and wash cloths, handkerchiefs, eating utensils, pencils and similar objects are dangerous after having been used by persons suffering from diseases of the intestinal tract (*e.g.*, typhoid fever or dysentery), of the skin (*e.g.*, erysipelas, boils, fungus infections), or of the respiratory tract (*e.g.*, diphtheria, scarlet fever and pneumonia). Venereal diseases may likewise be transmitted by these objects, although this is rare. Public toilet seats, drinking cups, hair brushes, etc., are always to be avoided.

The more recent the contact of fomites with infectious sources, the more likely are they to transmit disease. This is especially true of the diseases due to very fragile pathogens like gonococci, meningococci, pertussis bacilli and influenza bacilli. Organisms recently removed from a patient seem to be much more highly infectious and dangerous than those cultivated on artificial media, but this is not always so, and many laboratory infections have occurred from established cultures.

*Insects which fly* from unsanitary, unscreened and undisinfected privies or hospitals to dwellings may mechanically transmit in-

\* Fomites are objects contaminated by patients with infectious disease; for example, dishes, pencils, handkerchiefs, bed linen, etc.



testinal and other disease organisms on their feet and bodies. In areas where flies abound, especially rural or city slum areas, if there is access to infectious sewage or feces, enteric fevers are likely to be more prevalent, especially during the summer months when flies are numerous. In places where city sewerage systems are not available, fly-borne disease can be avoided to a large extent by the construction of screened and deep-pit or other sanitary types of privies or, better still, by the installation of sanitary plumbing and septic tanks. Plans and specifications for such structures can be obtained from State Health Departments.



Fig. 135.—Scalding after dishwashing—the safe way. (Photo by Lewis Hine. Courtesy of Cleanliness Institute.)

It is clear that to avoid the mechanical transmission of disease, objects like clothing and dishes, likely to be contaminated with discharges from a patient or carrier, should be carefully handled, segregated and boiled or steam pressed, or rinsed in solutions of disinfectants, or thoroughly sunned. Restaurant proprietors who have the well-being of their patrons in mind, either carefully scald all dishes after washing them (Fig. 135) or, after thoroughly washing them in *hot*, soapy water, rinse them in clean water containing *at least 100 parts per million* of available chlorine, and dry by drainage (Fig. 136). The odor of chlorine around a lunch counter

is a favorable sign! A good deal of attention has been given this point recently, and health departments are requiring better practices at cafes, soda fountains and lunchrooms.<sup>1, 2, 3</sup> Some of them are frightful! For example, in one city which is representative of most, public drinking glasses and rinse-waters at soda fountains, cafes and beer saloons were examined for total numbers of bacteria, hemolyzing forms, streptococci, colon-aerogenes organisms and the organisms of Vincent's angina. Swabs moistened in sterile water are used to remove the bacteria from the dishes and the water, squeezed from the swabs, is used to inoculate broth or agar plates.<sup>4</sup> Plate counts on the rinse waters ran into the millions per cc., with many thousands of colon organisms. On the glasses themselves the

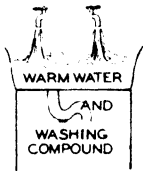
### UTENSIL WASHING

All food and drinking utensils must be cleaned and disinfected before each use.

THEY SHOULD BE

1. WASHED

IN



2. RINSED

IN



3. DIPPED

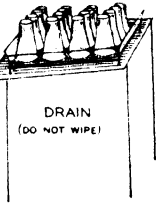
IN A CHLORINE SOLUTION



ONE TEASPOONFUL OF ANY ONE OF THE COMMERCIAL ALKALINE BLEACHES TO ONE GALLON OF WATER

4. DRAINED

FOR ONE MINUTE



NOTE--SCALDING WATER (180 F OR MORE) MAY BE USED INSTEAD OF THE CHLORINE SOLUTION.

Fig. 136.—Diagram showing steps in proper cleaning and disinfecting of soda fountain glassware. (Bureau of Food Control, Baltimore City Health Dept.)

same organisms were found and, in addition, hemolytic streptococci and Vincent's angina organisms. Invisible virus particles of influenza were probably present also. The horrors of the soda fountain and cafe are none the less real because sometimes hidden! There is no exact method of measuring the amount of disease spread by dirty dishes and eating utensils in restaurants. Probably we all have a good deal of immunity to the bacteria so transmitted. However, from an esthetic viewpoint one does not like to feel that a little saliva from previous patrons is being included, gratis, with his meal.

**Paper Dishes.**—The use of paper cups, dishes and eating utensils is the best step toward eliminating the sanitary evils of public glass

and chinaware and metal spoons and forks. Not only is expensive dishwashing equipment, with its noise, sloppiness and heat eliminated, but labor and fuel costs are reduced, breakage costs are trifling and esthetic and sanitary standards enormously improved. Bacteriological studies of paper used for containers and tableware show that the processes used in their manufacture result in a product with negligible content of microorganisms (Fig. 137).<sup>5,6,7,8</sup>

Milk supplies and the food in any kitchen may become infected from the hands of careless milkers, dairymen or cooks who are



Fig. 137.—Drawn from experiences during which ordinary safeguards of health were temporarily in abeyance and when transmission of disease by fomites was an ever present danger. The sanitary and mechanical advantages of paper dishes, cups and eating utensils are well illustrated here. (The Public Health Committee of the Paper Cup and Container Institute, New York.)

carriers of disease germs. Washing the hands after defecation or urination is a partial safeguard against transmission of intestinal disease in this way, but careless and ignorant persons are often very lax in this respect. Pasteurization of milk and the thorough cooking of foods are other safeguards. Persons who handle foods for restaurants or institutions, as well as dairy workers, should be required by law to pass bacteriological examinations and are so regulated in many communities, but enforcement is often lax.

**Transmission by Droplets of Saliva, Mucus, etc.**—The *secretions of the nose, throat, mouth and lungs, all combined to some extent with*

*saliva, constitute one of the most formidable vectors of disease.* Pneumococci, streptococci, meningococci and tubercle bacilli, as well as other organisms of respiratory disease, are thus transmitted. Saliva alone is of the greatest importance in disease transmission. We are all very careless in our habits in regard to saliva, far more so than we like to realize. The case has been stated vividly by a famous physician: "If infection by contact is of such very great importance in the fecal-borne diseases, how much more important must it be in diseases in which the infective agent is found in the secretions of the nose and mouth, as is the case with diphtheria, scarlet fever, smallpox, mumps, measles, whooping cough, tuberculosis, influenza, and cerebrospinal meningitis. Every one avoids feces and urine, but it is only the very few who have any objection to saliva.

"Not only is the saliva made use of for a great variety of purposes, and numberless articles are for one reason or another placed in the mouth, but for no reason whatever, and all unconsciously, the fingers are with great frequency raised to the lips or to the nose. Who can doubt that if the salivary glands secreted indigo the fingers would not continually be stained a deep blue, and who can doubt that if the nasal and oral secretions contained the germs of disease, these germs would not be almost as constantly found upon the fingers? In this universal trade in human saliva the fingers not only bring the secretions of others to the mouth of their owner, but there, exchanging it for his own, distribute the latter to everything that the hand touches. This happens not once, but scores and hundreds of times during the day's round of the individual. The cook spreads his saliva on the muffins and rolls; the waitress infects the glasses and spoons; the moistened fingers of the peddler arrange his fruit; the reader moistens the pages of his book; the conductor his transfer tickets; the lady the fingers of her glove. Every one is busily engaged in this distribution of saliva, so that the end of each day finds this secretion freely distributed on the doors, window sills, furniture and playthings in the home; the straps of trolley cars; the rails and counters and desks of shops and public buildings; and, indeed, upon everything that the hands of man touch. Besides the moistening of the fingers with saliva and the use of the common drinking cup, the mouth is put to numberless improper uses which may result in the spread of infection. It is used to hold pins, string, pencils, paper and money. The lips are used to moisten the pencil, to point the thread for the needle, to wet postage stamps and envelopes. Children 'swap' apples, cake

and lollipops, while men exchange their pipes. Sometimes the mother is seen 'cleansing' the face of her child with her saliva-



Fig. 138.—*Upper*, Unstifled sneeze explodes a cloud of highly atomized bacteria-laden droplets. Some droplets travel at such high speed that they are streaks even at  $1/30,000$  of a second. *Lower left*, Stifled by handkerchief, only few sneeze droplets escape to infect the atmosphere. *Lower right*, Stifling by hand, although less effective than handkerchief, stops most droplets. (Courtesy of M. W. Jennison, Depart. of Biology, Massachusetts Institute of Technology.)

moistened handkerchief, and perhaps the visitor is shortly afterward invited to kiss the little one" (Chapin).

Droplets of saliva are presumably responsible for much disease transmission. Influenza, infantile paralysis, and other diseases are probably transmitted by such means. Every cough or sneeze results in a germ-laden spray. Sneezing or coughing in public without a handkerchief is reprehensible but commonplace (Fig. 138). The droplets may remain suspended for some time in the air and may be carried many feet by draughts. The droplets may become dry; the bacteria, which constitute what are called *droplet nuclei*, then float about through the air like dust particles.

**Transmission by Dust.**—Little imagination is needed to understand how disease may be transmitted by dust. Particles of saliva or sputum containing bacteria as a nucleus (droplet nuclei) fall to the floor or ground, dry quickly and, if not exposed to excessive heat or sunlight or other unfavorable influences, the bacteria in them may survive for considerable periods. When the dust is stirred up, persons inhaling it or getting it into operative or accidental wounds may suffer an attack of disease. Probably the respiratory diseases like tuberculosis, pneumonia, diphtheria and scarlet fever are sometimes transmitted by such means, since the organisms involved resist drying. A good example of what is probably a dust-borne disease is San Joaquin Valley fever (see section on fungi, page 200). The dust in places where psittacine birds (parrots, etc.) are raised and sold was formerly a source of much infection with the virus of "parrot fever" (psittacosis), since the virus occurs in feces and nasal secretions of infected birds. Regulations have done much to reduce this infection.

**Transmission by Foods Other than Milk.**—*Meats.*—The flesh of hogs and cattle is sometimes infectious because the animals from which it comes were diseased. As shown by Scherago and his colleagues, *Salmonella* organisms are not infrequent in market meats, both pork and beef.<sup>8a</sup> Undulant fever organisms are probably not infrequent in both of these foods, since swine and cattle may be infected. Persons handling or eating raw, fresh meats are especially liable to these infections.<sup>8b</sup> Bovine tuberculosis is now rare in the United States, thanks to the U. S. Department of Agriculture and state agricultural institutions. Undulant fever is decreasing due to efforts by these same organizations. The transmission of "rabbit fever" (tularemia) by the flesh of wild rabbits has received so much newspaper comment that it should be a matter of common knowledge. Marketers of wild rabbits, as well as their customers, have furnished case stories for many a newspaper reporter.

The obvious means of avoiding these meat-borne diseases is to

*eat only thoroughly cooked meats.* Market meats from reputable dealers have usually remained long enough in cold storage for danger of infection to diminish somewhat, but not entirely. Inspection of meats and slaughter houses by U. S. veterinarians excludes much infectious meat from the market. "Black market" meat, being under no official supervision, is especially dangerous. After handling *any* raw meat the hands should be thoroughly washed in soapy water.

*Vegetables.*—In the United States it is reasonably safe to eat raw vegetables such as lettuce, celery, chicory and other "salad greens" because human excrement is not used as fertilizer. In the Orient and some other countries human manure is widely used. Our troops in North Africa and Mediterranean countries and the Orient were faced with this problem. Even at home vegetables may transmit disease.<sup>9</sup>

The only sure means of avoiding infection with intestinal pathogens from this source is to refrain from eating in a raw state vegetables which grow in intimate contact with the soil or which cannot be well washed and peeled or shelled. The chief offenders are those mentioned above. If one has facilities, he may operate his own kitchen garden under proper sanitary supervision.

*Cooked Foods.*—We have stated that cooking meats, vegetables and other foods eliminates infection initially present in or upon them. But infection may be unwittingly introduced after cooking by unsuspected human carriers, particularly of intestinal pathogens, sometimes streptococci as shown by Getting, Foley, *et al.*,<sup>10, 11, 12</sup> and staphylococci.<sup>13</sup> Foods left uncovered are subject to infection from the urine and feces of rodents which carry *Salmonella* species, especially *S. typhimurium*. According to Welch, Ostrolenk, and Bartram,<sup>14</sup> only a small percentage of rodents naturally harbor *Salmonella* (paratyphoid and food infection) whereas Stone<sup>15</sup> has shown that human beings not infrequently harbor them, as well as *Shigella* (dysentery), and *Eberthella typhosa*. If the food is left unrefrigerated for several hours these organisms can grow. Many a food-infection outbreak after church suppers, picnics, etc., follows such a train of events.

**Transmission by Direct Contact.**—This means of disease transmission needs little comment. Obviously, if one rubs against infectious material he runs a risk of infection. Usually it is easy to guard against such an eventuality, especially if one avoids transferring the *contagium* to its special portal of entry. One does not voluntarily come into physical contact with feces, sputum or the visible sores

or pustules of infected persons. However, an innocent kiss may transmit tuberculosis, pneumonia, scarlet fever, diphtheria and other diseases. The best examples of contact-transmitted disease are syphilis and gonorrhea, both spread by coitus, the former by kissing also.

### BIOLOGICAL TRANSMISSION OF DISEASE

**Biting Insects.**—Many bacteria are associated with insects.<sup>16, 16a</sup> There are several diseases the sole natural means of transmission of which is the bite of insects. The classical observations of Smith and Kilborne in 1893 on transmission of Texas fever of cattle by the cattle tick (*Boophilus annulatus*) were the first report on tick transmission of disease, although certain mosquitoes and other insects had previously been found to transmit other parasites (malaria, filaria worms, etc.). Usually, but not always, each disease has its own specific insect vector. Malaria is the best known disease of this type, being carried only by certain mosquitoes (*Anopheles*). Dengue fever, yellow fever, African sleeping sickness, typhus (not typhoid), Rocky Mountain spotted fever, tularemia, and some other diseases, are also transmitted by the bites of certain specific insects (mosquitoes, ticks, lice, fleas). Plague is an outstanding example of a disease transmitted by biting insects (rat fleas) and has played an important role in the history of nations. Tick-borne diseases, like Rocky Mountain spotted fever and possibly tularemia, transmitted among rabbits and from them to man, may be carried by birds, as rabbit ticks have been found making long journeys attached to birds.

In some cases the agents of disease are ready to infect at all times after they enter the body of the insect, as in plague or tularemia. In other cases the bite of the transmitting insect is not dangerous until after an interval of from a few hours to 2 weeks, during which the disease agent undergoes some sort of developmental or activating cycle inside the insect, the so-called extrinsic incubation period. This is well illustrated by malaria and typhus fever. In yellow fever, on the contrary, the mosquito contains infectious material at all times after his first infectious blood meal, as can be shown if the whole insect is ground up in serum and injected. However, the bite is not infectious for about 10 to 12 days after taking an infectious blood meal. Apparently the virus does not undergo any alteration in the mosquito which causes it temporarily to lose its infectivity, but it seems to require about 10 days to migrate from the stomach to the biting apparatus.



**Insect Feces.**—Cockroaches were shown by Chambers as early as 1914<sup>17</sup> to transfer cholera vibrios in their intestines for at least 48 hours after feeding on human cholera feces. Ants transmit the disease, and possibly others, in the same manner. Flies have long been under indictment for the same crimes.<sup>17a</sup>

The feces of lice infected with typhus organisms will infect if scratched into the skin. Feces of infected fleas contain plague bacilli and may contaminate small wounds or scratches. Indeed, many blood-sucking insects may pass infective agents in the feces and may also cause infection by being crushed on the skin near, or in, an abrasion or wound. Engorged ticks are especially dangerous in this respect because they contain a relatively large volume of blood. It seems not unlikely that flies may also transmit infantile paralysis by fecal contamination, as the virus has been demonstrated to occur in flies. However, there is no direct proof that feces of flies contain the virus.<sup>18, 18a</sup>

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and plant species, and are used in the analysis of the living cell as to its component proteins or protein-complexes, in the study of infection, disease, crime, ethnology, botany, zoology, various substances and processes used in industry, and for many other purposes related not only to bacteriology but to other branches of science as well. The methods of immunology are so widely used as laboratory tools and are based on such fundamental biological phenomena that one must have a clear grasp of the underlying principles before a detailed study of bacteriology will prove profitable.<sup>1, 2, 3</sup>

**Blood.**—Before discussing the nature of immunity and the methods of studying it, it will be advisable to devote a little space to a description of blood and its constituents, since these substances are much used in immunological investigations.

The blood is conveyed by the circulatory system to the remotest vascular parts of the body where it carries food to the tissue cells and removes their waste products. Blood may carry death and destruction to certain body cells if toxin-producing bacteria gain a foothold somewhere in the body and secrete their poison (toxin) into the tissues, where it is absorbed by the blood and lymph. On the other hand, blood carries with it at all times substances and special cells which help to combat bacteria and toxins. The study of these protective substances and cells is part of the science of *immunology* and a knowledge of them is important to a clear understanding of the way in which bacteria causing disease react in the body.

**Important Blood Constituents.**—For the purposes of this discussion blood contains four important constituent parts. First, there is the *plasma*, the yellowish, transparent, fluid part of blood, consisting of a solution of proteins, salts and other soluble substances as well as food for, and wastes from, the body cells. The plasma has in solution, also, the substances necessary to the formation of a *clot* or *fibrin*.

**Fibrin Components.**—When freshly drawn blood is allowed to stand in a test tube or flask, it will be found, after a few minutes, to have solidified or clotted. The material responsible for this forms a fine fibrous *network* in the blood after the blood leaves the body. The meshwork is composed of a substance called *fibrin*, a highly complex protein which is very elastic. The fibrin clot soon shrinks to about half the original volume of the blood, squeezing out of its meshes the fluid portion of the blood, much as a wet, contractile sponge would exude water. Most of the blood cells are caught and held in the fibrin (Fig. 139). Associated with fibrin production are

small, stellate, non-cellular bodies called *platelets*. Their nature is not yet fully understood (Fig. 140 *F*).

*Serum*.—The fluid portion of blood, after it is separated from the fibrin, is called *serum*. Since fibrin usually enmeshes the blood corpuscles, serum looks yellowish and transparent, like plasma.

*Erythrocytes*.—In the plasma, before clotting, are suspended the red corpuscles (or *erythrocytes*), non-nucleated cells (when mature) which give to blood its opacity and red color and which carry oxygen from the lungs to the tissues, and carbon dioxide from the

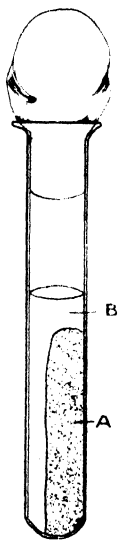


Fig. 139.—Tube with clot (*A*) which has shrunk, enmeshing the blood corpuscles and exuding clear serum (*B*).

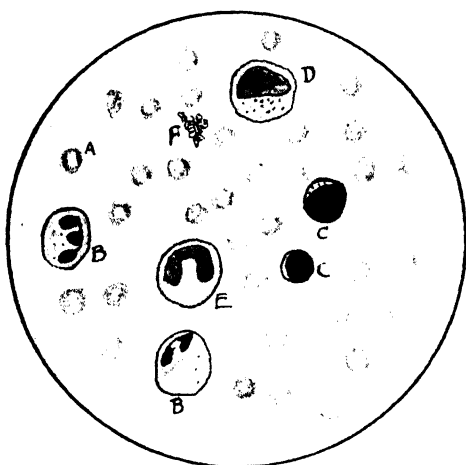


Fig. 140.—Stained smear of normal human blood showing different kinds of corpuscles. *A*, red corpuscles; *B*, white corpuscles (polymorphonucleated leukocytes); *C*, small lymphocytes (another kind of leukocyte); *D* and *E*, other kinds of leukocytes (large lymphocytes). *F*, Blood platelets.  $\times 900$ .

tissues to the lungs. The color of erythrocytes is due to the red substance, *hemoglobin*, which they contain. When they are ruptured by plasmolysis or other means, the hemoglobin is released and the plasma becomes transparent and red. The cells are said to have *hemolyzed*. Certain bacteria (*e.g.*, beta-type hemolytic streptococci and *Bacillus subtilis*) are very active in producing hemolysis.

*Leukocytes*.—In addition to erythrocytes blood contains colorless, ameba-like cells called *leukocytes* ("white cells") or *phagocytes* (*phago* = eat; *cyte* = cell). These are relatively large cells which

have a definite nucleus and means of locomotion like amebae. They act as scavengers and "policemen" in the blood (Fig. 140 *B, C, D, E*). Like amebae, leukocytes can ingest food particles, and in the body they serve by ingesting and destroying any foreign particles, such as bacteria, dead body cells and the like, that may gain entrance to the tissues or blood stream. The leukocytes can leave the blood vessels and congregate in the tissues wherever any irritation exists. Centers of inflammation such as the bacteria in a boil, or a splinter of wood or other foreign particle, exert a strong attraction for leukocytes, which concentrate in such areas. The nature of this attraction is not fully known. It is called *chemotaxis*. The white cells try to dispose of the foreign particles by ingesting them and also by giving off enzymes which will kill and destroy or digest them. Often many leukocytes are in turn killed by poisons of the bacteria they ingest. Thus, the white, creamy material in a boil or other infected lesion or around a "fester" splinter is made up largely of dead and living white corpuscles, dead and living bacteria, tissue debris, lymph, fibrin and serum. It is called *pus* and the dead leukocytes in it are called *pus* cells.

**Lymph.**—Lymph is very much like blood which has been deprived of its corpuscles by passing through the thin walls of the smaller blood vessels as through a fine filter. The fluid thus seeping out of the vessels travels slowly in the fine spaces surrounding the blood vessels and other organs and eventually collects from all parts of the body in a large drainage vessel and is returned to the blood stream.

**Foreign Substances.**—As mentioned above, the phenomena of immunity depend on the reaction of the animal body to foreign substances which gain entrance to it. For the purposes of this discussion, the interior of the gastro-intestinal tract is considered *external* to the body proper, as it is open to the outer world at both ends and has no direct gross connection with the principal body cavities. We shall speak, therefore, only of foreign substances which get into the body *parenterally* (i.e., other than through the gastro-intestinal tract). This includes, generally, substances or bodies injected into the tissues with hypodermic needles, or introduced by means of splinters or wounds, or forced into the peritoneal or pleural cavity, or released or absorbed from mucous and other surfaces into the blood, or in any situation where they are not exposed to the digestive enzymes of the mouth, stomach or intestines.

**Reaction to Foreign Substances.**—When foreign substances are

solid, inert and insoluble particles like wooden splinters, shrapnel fragments, sand or glass, the tissues respond locally with inflammation or pus formation to expel them or walls of fibrous connective tissue to incarcerate them, but there is no demonstrable permanent general change in the chemical or physical composition of the blood or tissues. When the foreign substances happen to be irritating microorganisms, however, or species giving off poisons, such as bacteria causing typhoid fever or diphtheria, the tissues respond not only locally with inflammation and other changes, but the whole body may be aroused and the tissues everywhere, and the blood, undergo a marked change in chemical or physical constitution, or both, of such a nature as to repel and destroy the irritating microorganisms, and their poisonous metabolic products (toxins) if any. The reaction is often so violent as to cause fever. The infective agents tend to die out, and the patient recovers but is "a changed man."

**Immunity.**—In diphtheria, whooping cough, typhoid fever, etc., the patient is so changed that he may never again, under *ordinary* circumstances of infection acquire the disease. He is said to be *immune* to that disease. Upon proper tests, his blood is found to contain substances which neutralize the toxins or unfavorably affect the bacteria, or both, and so protect him from the disease, and his tissues are found to be quite unaffected in any *visible* way by the injection into them of the living organisms or their poisons.

In some cases, as under experimental conditions, foreign organic substances of a harmless nature may gain entrance to the tissues. If these are soluble proteins, for example proteins of harmless bacteria, egg white, or animal serum, the body reacts in much the same way as toward bacterial toxins or the proteins of infecting organisms, *i.e.*, durable changes occur in the tissues which are directed toward the destruction and removal of the foreign substance in question. Therefore, while much of the discussion which follows will deal with infectious bacteria, because much of our knowledge of the subject of immunity is derived from the researches of bacteriologists interested in immunity to disease (notably Pasteur), the principles involved must be thought of in a broad biological sense as applying generally to foreign substances (usually proteins) of diverse origins.

In man and animals, immunity from various diseases is usually acquired as a result of having those diseases and recovering from them. This is called *naturally acquired immunity*. Common examples of naturally acquired immunity are resistance to diphtheria,

typhoid fever, measles and smallpox in persons who have recovered from them. In all such instances there are formed, by the living cells of the body, certain substances which are excreted into the blood and which neutralize the toxins produced by the toxigenic organisms. Other substances formed by the tissues destroy the bacterial cells and help the leukocytes to destroy them. Any *soluble foreign protein*, and certain carbohydrates, will stimulate the production of such substances.

**Antibodies.**—All such protective substances are called *antibodies*. Often antibodies continue to be produced by the body cells long after the stimulus which excited their production disappears, and it may be to this that prolonged immunity, such as follows mumps, measles and diphtheria, is due. However, it must also be emphasized that immunity often seems to be dependent on certain properties of the tissue cells which are not related to the presence of *demonstrable* antibodies in the blood stream. Conversely, the presence of certain antibodies easily demonstrable in the blood does not imply or guarantee immunity.

**Specificity of Immunity.**—Immunity is said to be *specific*. The antibodies associated with immunity are also specific. That is, a person who recovers from diphtheria is immune to diphtheria. He is not, however, necessarily immune to other diseases, such as typhoid fever or whooping cough. Not *every* disease confers immunity. Some which do so have been mentioned. Pneumonia, influenza and erysipelas are a few of the diseases that do not confer any very definite or prolonged immunity and of which second attacks are frequent.

In the case of nonbacterial proteins, the principle of specificity holds with equal regularity. Egg-white antibodies will not act against hookworm proteins, or horse-serum antibodies against rabbit serum.

This specificity is dependent on the chemical and physical structure of the antibodies involved, a matter which will be discussed farther on. It is closely related to the chemical and physical structure of the foreign substance stimulating the formation of antibodies.

**Antigens.**—It is clear that the entrance of a foreign substance of definite chemical nature into the normal body parenterally stimulates the production of antibodies against that particular foreign substance. We have seen also that the antibodies produced are specific. This is one aspect of a general biological phenomenon. Almost any *protein*, and a few carbohydrates, soluble in water, will

stimulate the production of antibodies against themselves when injected into any animal species other than that from which they came. Such proteins or carbohydrates are spoken of as *antigens* (*anti* = against; *gen* = produces). These, like antibodies, are specific. That is, each pure antigen engenders only one sort of antibody, and will react *only* with that antigen. We therefore say that *antigen-antibody reactions are highly specific*. The antigen-antibody reaction, accordingly, gives us a method of high precision in the study of proteins. Several methods of using this instrument in microbiology will be discussed after we give some attention to the nature of specificity.

**Specificity of Antigens.**—It is very simple to state that antigens and antibodies are *specific* for each other, but the explanation is far from simple. However, several pertinent facts may be of help in forming a concept of the relationships.<sup>4, 5</sup> First, specificity is a matter largely of chemical and physical structure. For example, a synthetic "protein" antigen (of which several have been prepared experimentally) having a given chemical structure (*e.g.*,  $\text{NH}_2\text{-C}_6\text{H}_4\text{-AsO}_3\text{H}_2$ . Protein) will, upon injection into the body, engender antibodies in the serum which react *only* with that compound. But if by chemical treatment we substitute a chemically altered antigen (say with an  $\text{SO}_3\text{H}$  group in place of the  $\text{AsO}_3\text{H}_2$  group), no reaction with the original antibody occurs. Almost any sort of chemical alteration in a protein will alter its specificity. Hydrolysis or destruction of solubility is likely to result in complete loss of antigenic properties. A slight reaction may occur if, instead of substituting an  $\text{SO}_3\text{H}$  group, we introduce, say, a Cl atom in place of the  $\text{NH}_2$  group. Then the antibodies produced in response to the original antigen are said to *cross-react* with the chlorinated antigen. Many cross-reactions occur in nature between closely related antigens.

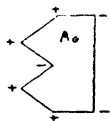
As a corollary of chemical structure, we may postulate certain definite distributions of positive and negative electrical fields of force on the surface of the colloidal particles or molecules which constitute antigens, due to electron arrangements upon the constituent atoms. Any change in chemical constitution of the antigen, such as introduction of a Cl atom or  $\text{SO}_3\text{H}$  group, will affect the distribution or relative intensities of these electrical fields or charges on the antigen molecule surface.

Now an antibody molecule is also a complex, colloidal, protein molecule and we may imagine its surface to have a pattern of electrical fields, positive or negative, analogous to those of the antigen, but *arranged as a mirror image* of those of the antigen. These elec-

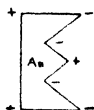


trical charges on the molecules are of the utmost importance. Not only do they determine the "energy-pattern" of the molecules, but the colloidal particles of either antibody or antigen are maintained in suspension-solution partly by the unneutralized portion of their electrical charge and partly by their small (colloidal) size.

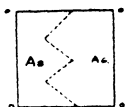
When mixed in a test tube, the antigen and antibody colloids coming into contact could, according to this hypothesis, orient themselves with respect to their positive and negative charges so that an absolute "fit" is obtained. Thus, we may imagine an antigen (*Ag*) to be represented by the figure:



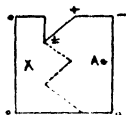
its corresponding antibody (*Ab*) by the figure:



and the antigen-antibody combination by the figure:



The compound colloidal particle forming upon reaction of these two would be an *electrically neutral* and *very large* one and, as a result, would become highly unstable in solution (a lyophobic colloid) and would therefore go out of solution and, in a test tube reaction, would become *visible* as a cloudy *precipitate* (Fig. 141). We should have what is known as a "*precipitin reaction*." A weak cross-precipitation might occur between antigen *Ag* and some antibody (*X*) closely similar to *Ab*, but not exactly like it:



This specific physicochemical interrelationship between an antigen and its antibody is commonly likened to the relationship between a lock and key. Some locks are absolutely specific for their

own keys. This may be altered by changing the internal structures of such locks. They may then open to several other keys of somewhat similar structure. It will be noted that we have to depend on analogies and speculation to some extent to explain the observed facts.

A few additional points concerning antigen-antibody reactions are worthy of mention at this point. First, the union of the two is firm but not always irreversible. They may be separated by certain procedures, showing that the reaction is largely physical and not chemical. Second, surface structures are in great part the determining forces. It is the arrangement of the *surface* molecules, and charges of the *surface* colloids which dominate specificity (Fig. 142).

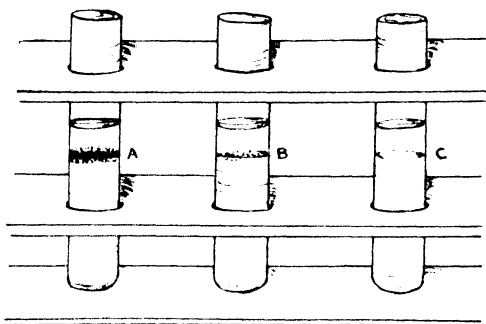


Fig. 141. Precipitin reaction between an anti-cow-protein serum and its antigen. The precipitin-containing serum is in the lower part of the tubes and the antigen has been placed above it in such a manner that the two are in contact but do not mix extensively. A strong reaction is seen at A. At B, the cow-protein antigen has been replaced by goat antigen and a very weak cross-reaction is seen. At C the antigen has been replaced by saline solution and no precipitate is seen at the junction of the two fluids. This is a "control" tube.

Third, these reactions proceed in two stages: (a) *combination* which may proceed rapidly at temperatures around 37° C.; (b) *visible precipitation*, cell lysis or other effect, which often occurs slowly and may often be demonstrated best after twelve to eighteen hours in the cold, at 4 to 6° C. The presence of electrolytes in the solution is usually necessary for the latter stage.

**Complexity of Natural Antigens.**—By means of synthetic chemistry and the precipitin reaction, it has been possible to delve deep into the physicochemical relationships of antigens and antibodies and the reactions of immunity. This has involved the study of very pure substances. In nature, antigens seldom occur as pure compounds and this greatly increases the difficulties surrounding

the study of antigen-antibody reactions involving such antigens as bacterial cells. A cell may consist of many antigens (*e.g.*, cytoplasm, flagella, capsule, etc.). The serum of a person or animal, following injection with such bacteria, contains a mixture of antibodies active against each separate antigen.

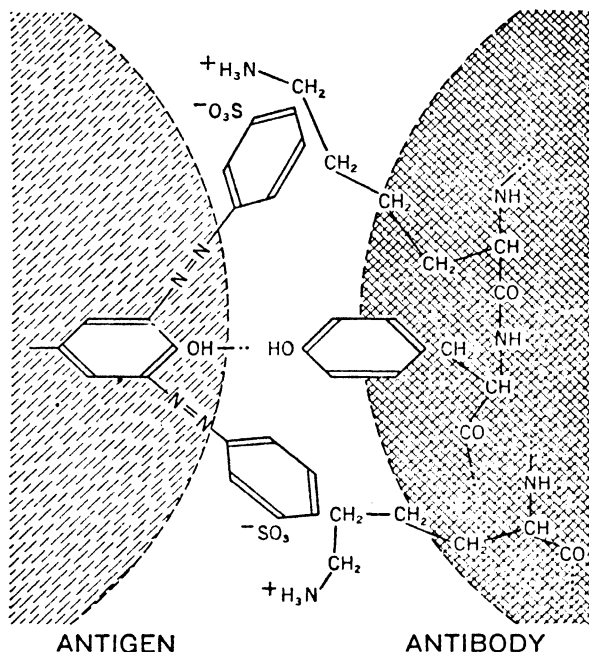


Fig. 142.—Hypothetical manner in which surface structure of antibody molecule might correspond to part of the surface of a synthetic conjugated antigen, in this case a native protein coupled with diazotized metanilic acid (Haurowitz).

To complicate matters further, the cells of two different, but related, species may contain certain antigens in common so that antisera will cross-react with both kinds of cell.

For example, three related species of dysentery bacilli may each contain four antigens as follows:

Species I	<u>A/B/C/D</u>
Species II	<u>C/D/E/F</u>
Species III	<u>E/F/G/H</u>

Obviously, upon injecting species I into a rabbit, antibodies a, b, c, d, will be engendered. Upon injecting species II into another rabbit, antibodies c, d, e, f, will be called forth. Likewise, species III will stimulate production of antibodies e, f, g, h. Now, the serum of rabbit I will react best of all with bacterial species I when these bacteria and serum of rabbit I are brought into contact. Serum II will similarly react best with species II, and serum III with species III. However, since serum I contains antibodies c and d, it will cross-react to some extent with bacterial species II, since the latter has these antigenic components in common with species I. There will be no cross-reaction between serum I and antigen III, but serum II will cross-react with species III. Such cross-reactions are commonplace and are the basis of recently investigated possible methods of producing immunity to disease by injections of non-specific antigens.<sup>6, 7, 8, 9, 9a, 9b, 9c</sup>

**Antibody Adsorption.**—If a given volume of serum I (say 5 cc.) be mixed with a heavy suspension of cells of bacterial species II, then antibodies c and d, and antigens C and D will combine, leaving antibodies a and b still free in the serum. By means of centrifuging, the bacteria with their attached antibodies c and d can be removed, leaving serum I free from antibodies c and d and *specific* with regard to species I; *i.e.*, it will no longer cross-react with species II and will react only with bacteria containing antigens A and B. If we further adsorb antibody b by treatment of the serum with some species having only antigen B in common with species I, then we obtain a pure A serum. Many such pure and specific sera are thus prepared and it has been possible to make very extensive analyses of the antigenic structure not only of bacteria, but of antigens from higher plants and animals, and other antigenic substances and to detect antigenic relationships hitherto unsuspected.

**Antigenic Structure of Bacterial Cells.**—Relatively little is known of the localization or nature of the different antigenic components of most living cells. With respect to bacteria, we know that certain antigens associated with the cell itself are quite widely distributed in certain groups. Thus, certain of the cell-body or somatic antigens (so-called O antigens) of many of the typhoid-paratyphoid organisms are shared in common. The antigens in the flagella (the so-called H antigens) are not so widely shared as a rule, but are more specific to the type or species.\*

In those species which possess capsules, the capsular substance

\* Except in certain phases of variation called the "group phase" when they become more like O antigens.

dominates the surface and often determines antigenic specificity of a very high order.

The localization of antigenic specificity in the capsular substance of such organisms as influenza bacilli, streptococci and Friedländer's bacillus is a striking phenomenon. Stripped of this specific surface antigen, the exposed proteins of the naked cells cross-react very widely with antisera of related and often unrelated species. The somatic or cellular antigens of all pneumococci are immunologically alike. Antibodies for one react equally well for all. But the antibodies of the capsules are not all alike and it has been found that there are at least thirty-two different kinds of pneumococcus capsular antigens. Each determines a different type of pneumococcus. This is part of a general biological phenomenon. Since the hypothetical electrical fields of the antigens and antibodies are active principally at the surfaces, specificity or nonspecificity or other antigenic or immunologic behavior may be thought of as depending upon whatever antigenic component of the cell dominates the surface at the time of the reaction. This may be greatly affected by various factors such as variation and chemical treatment.

**Types of Antibody Response and Reaction.**—There are different manifestations of antibody action, some of them demonstrable in test tubes as visible reactions, some of them not visible except indirectly by secondary tests for their presence or absence, such as complement fixation or animal protection tests. The different manifestations of antibody action are loosely spoken of as though different antibodies were involved, but the multiplicity of antibodies may be more apparent than real. The kind of antibody action manifested seems to depend in great part on the kind and size of antigen molecule or cell, the physical conditions of the suspending fluids, electrolytes, and other factors. For convenience, we shall speak of "types of antibody," meaning thereby "types of antigen-antibody reactions." Among them are antitoxins, cytolsins, agglutinins and precipitins.

**Antitoxins.**—When bacteria gain a foothold in the body and secrete toxin into the blood, the toxin, being protein-like, stimulates the production of an antibody. This antibody neutralizes the toxin and is therefore called *antitoxin*. The reaction may be thought of as resulting in precipitation in which the particles of precipitate, as a rule, are too small to be seen. Under certain conditions in which the quantitative relations are carefully adjusted visible minute flakes or floccules are produced (see below). The reaction is

quantitative but seems to obey the laws which govern physical adsorption rather than chemical combination.

**Quantitative Relations.**—As mentioned above, when exactly the right proportions of toxin and antitoxin are brought together in a test tube a visible precipitate or flocculation occurs. This fact is made use of in determining the concentrations or “strengths” of toxin or antitoxin. For example, we may set up a row of ten tubes. In the first we will put serum (Fig. 143) containing a quantity of antitoxin arbitrarily spoken of as 2 units.\* In the next we place 4 units, in the next 6 units, and so on. We then add to each tube a fixed amount, say 1 cc., of filtered broth culture of *C. diphtheriae* which contains toxin in unknown amount. After a short time flocculation appears in one of the tubes, let us say the sixth tube. Since this contained 12 units of antitoxin, we have a measure of the potency of the toxin broth. We say that it contains 12 flocculation units (12Lf) of toxin per cc. This is an arbitrary unit of potency.

**Zone Reaction.**—This reaction illustrates another curious anomaly called a zone phenomenon (sometimes called a prozone). The reaction occurs *first* only in one tube, *not* representing the greatest or least concentration of reacting substances. Obviously if one substance is present in too great or too little concentration relative to the other, conditions are not optimal for a visible reaction. This is a general immunological phenomenon, and necessitates careful adjustments of quantitative factors in all such work.<sup>9d</sup>



Fig. 143.—Method of holding pipette in serological work. Tilting the index finger slightly admits air slowly and allows the fluid to run out of the pipette. (Courtesy of the Seminars, published by Sharp & Dohme, Inc.)

\* A unit (*approximately*) of diphtheria antitoxin is the least amount necessary to protect standardized (250 to 300 gm.) guinea pigs against 100 minimal lethal doses (M.L.D.) of diphtheria toxin. An M.L.D. kills about 75 percent of such pigs in from four to five days.

**Cytolysins and Complement.**—The proteins of which a given bacterial cell is composed may be different from the toxin (if any) formed by that cell and may stimulate the body to produce antibodies which assist in the dissolution (or *lysis*), by serum enzymes, of the bacterial cell itself. These antibodies are termed *cytolysins* (*cyto* = cell; *lysin* = dissolver). They are also called, because of the way in which they combine with the antigen and the serum enzymes, *amboceptors* or *sensitizers*. Their action is a little more complex than the simple neutralization of toxin by antitoxin and may be described briefly as follows:

The cytolytic antibody first combines with the foreign cell that called it into being. The cell may be a bacterium, an erythrocyte or a cell of any other nature. This simple combination is, however, not sufficient to destroy the cell, and there is no visible reaction. A second substance, which is *normally* present in all mammalian blood and which is entirely *nonspecific*, is necessary to *complete* the lytic action. It, too, combines with the cell which, in order for it to act, must already have been *sensitized* by the *sensitizer* or *amboceptor*. *Lysis*, or cell dissolution, then results.

The term *amboceptor* (*ambo* = two-sided; *ceptor* = holder) is derived from the hypothetical idea that the cytolytic antibody combines with the cell (antigen) on the one hand, *sensitizing* it to the action of the complementary substance (*complement*) with which it combines on the other hand. It is as though a thief (the antigen) were held by a policeman (amboceptor) with one hand, the policeman being unable to subdue the culprit until his other hand is equipped with his club (complement).

The latter substance is called *complement* because it is necessary to the *completion* of the lytic action. Another name for it is *alexin*. Complement cannot, *by itself*, destroy foreign cells, but must act through the intermediation of the amboceptor or sensitizer. The sensitizer is a *specific* antibody, but *complement is nonspecific*; it helps *any* sensitizer to complete its work. After complement has combined with the sensitized cell it is no longer active and is said to be "*fixed*." It is adsorbed.

**The Complement-fixation Reaction.**—This inactivation, or fixation of complement was first demonstrated in 1901 by Bordet, a famous Belgian scientist. It may occur in reactions which do not necessarily result in cell lysis. Being a colloidal substance, it is readily adsorbed on the surfaces of any finely divided particles, visible or invisible. For example, it is adsorbed by the flocculation resulting from the interaction of toxin and antitoxin as described

above. Complement may be fixed in precipitin, agglutination and toxin-antitoxin reactions but is not a necessary component of such reactions.

Bordet discovered that if the serum of a person who had recovered from bubonic plague (and therefore containing antiplague sensitizer) were mixed with plague bacilli, the free complement in the serum was all used up or fixed in destroying the bacilli. When the complement was tested for, by an appropriate method, none was to be found free in the serum.

This phenomenon of complement fixation, is used today by immunologists and bacteriologists, police detectives, chemists and industrialists to diagnose disease by the identification of certain antigens causing disease, or their corresponding antibodies which appear in the blood as a result of disease, or to identify blood stains, study proteins, investigate manufactures and the like. The several facts on which the complement fixation test is based may be recapitulated as follows:

1. Sensitizers combine *only* with their *specific* antigen.
2. Complement will combine with an antigen *only* when the latter is *sensitized*.

Therefore, if amboceptor, antigen and complement be mixed in a tube, we can determine whether antigen and amboceptor have combined by testing to see whether the complement has been fixed or not. If complement has been fixed, then we know that properly suited antigen and amboceptor were in the mixture. Knowing the identity of either antigen or sensitizer we can, therefore, identify the other.

**The Wassermann Test.**—A very common use of the complement-fixation test is in the diagnosis of syphilis. It was first described by Wassermann. It serves to illustrate the method, which is generally applicable. It may be explained as follows: The serum of the patient, which (we shall assume) contains the syphilis amboceptor, is mixed in a test tube with a little of the syphilis antigen. The usual combination occurs and some or all of the complement in the patient's serum is used up, or fixed. *The difficulty is in knowing whether the complement is fixed or not*, since all of the substances, and the reaction itself, are quite invisible, and one cannot see what is going on in the test tube. One must test the mixture to see whether there is any unfixed or free complement which could *complete some other reaction if it had the opportunity*. A very simple test is to add a few red blood corpuscles *which are already sensitized* to complement



with a specific red-cell-lytic amboceptor prepared for them. If there is any free or unfixed complement, it will combine with the red-corpuscle-amboceptor complex and dissolve the corpuscles. One can easily see if the red corpuscles have been dissolved, because the suspension of cells becomes clear and ruby-red, whereas previously it was cloudy red. It is the custom to destroy entirely the *varying* amounts of complement already in the patient's serum by gentle (56° C. for thirty minutes) heating (*inactivation*) and to substitute a standard, *carefully measured* (or titrated) amount of fresh, active complement from some other source, such as the serum of guinea pigs. This also enables the person doing the test to form some opinion as to how much complement remains free, since he knows how much he added originally. If part of it has been fixed, some of the sensitized red corpuscles will fail to be *hemolyzed*. *When all the cells are hemolyzed, it indicates that none of the complement was fixed by the patient's serum in contact with the syphilis antigen. It is then known that the patient did not have syphilis amboceptor in his serum.* The same method is employed to detect and study many other sorts of antigen and antibody. It is of particular interest in connection with viruses.

**Agglutinins.**—In addition to antitoxins, cytolysins and complement, there are antibodies which cause bacteria to gather and stick together in flocks or clumps as though they were coated with some glutinous substance. Such antibodies are called *agglutinins*. Like all other antibodies, they are specific. They do not necessarily kill bacteria but aid the leukocytes by gathering the latter's prey into groups. A leukocyte, or other phagocytic cell, can engulf 50 agglutinated bacteria fifty times as easily as 50 separate ones, and in much less time.

Agglutinins are very widely used in the identification of bacteria and the diagnosis of disease. Let us assume that a patient has a severe fever which has remained undiagnosed for a week or more. We draw a little blood from a vein and allow it to clot. We then remove the clear serum and mix it, suitably diluted (1:20; 1:40; 1:80; . . . 1:2560), in a series of test tubes with, for example, a suspension of typhoid bacilli (*Eberthella typhosa*). If the patient has typhoid fever, the serum will contain *typhoid agglutinins* and the bacilli will be found in flocks or clumps even in high dilution (Fig. 144). If the fever is due to some other organism (and assuming that no antigenic components of the latter organisms are shared in common with typhoid bacilli so as to cause cross-reactions) no antibodies capable of agglutinating typhoid bacilli will be present

and the typhoid bacilli will not be agglutinated. This means of diagnosing typhoid fever is called the *Widal reaction* after Widal, who first published upon the subject. The test as used by Widal is, however, subject to a number of serious errors and it is better to use the process of receptor analysis described later (see page 302).



Fig. 144.—Agglutination of bacteria by immune serum in dilutions of 1/10, 1/20, 1/50, 1/100 (*i.e.*, 1 part serum and 9 parts normal saline, and so on). The first tube is a control containing the bacteria in a 1/20 dilution of normal serum. Some of the organisms have settled to the bottom through the action of gravity, but enough remain suspended to render the fluid opaque. The immune serum agglutinates the bacteria completely in the middle tubes. The organisms have clumped and fallen to the bottom, leaving the fluid clear. The 1/200 dilution of immune serum in the last tube is too weak to agglutinate the bacteria. (From Hiss, Zinsser and Russell, "Text-book of Bacteriology," D. Appleton-Century Co., publishers.)

### Identification of Bacteria by the Agglutination Reaction.—

Conversely to the Widal test, in the identification of an unknown organism, sera containing various *known* antibodies are mixed with suspensions of the unknown bacterium, and agglutination is looked for. Suppose, for example, that we have a gram-negative rod which, by its cultural reactions, we know to belong to the typhoid-

dysentery group. We may, as a preliminary test, set up two series of tubes: A, containing serial dilutions of serum of a typhoid-immune animal; B, containing dilutions of serum of a dysentery-immune animal. A drop of our "unknown" bacterial suspension is added to each tube. If, after several hours, no change has occurred in the first series of tubes while the serum in the tubes of series B has caused the bacilli to fall to the bottom of the tubes in flakes or granules, we know that, since the serum in B contained only dysentery agglutinins, our unknown organism must be some species of *Shigella* (dysentery bacilli). Many other bacteria, saprophytic and parasitic, may be identified in this way. For very exact work it is advisable to use sera the specificity of which has been increased by the process of antibody adsorption previously described.

**Somatic and Flagellar Agglutinins.\***—It will be remembered that flagella stain differently from the bodies of bacilli. This suggests that flagellar protein differs in some way from body, or somatic, protein. It has been found that agglutinins (which are also referred to as *receptors*) produced by motile bacilli of the typhoid-paratyphoid group and other groups are of two sorts, flagellar and somatic. One sort, the so-called *H agglutinins* or receptors, are engendered by, and act only against, the protein present in the *flagella* (H protein). With a few exceptions (an important one being in the genus *Proteus*, in which the situation is reversed), the flagella of each motile species stimulate the production of agglutinins active against the flagellar protein *only* of that species.

The second kind of agglutinins, called *O agglutinins* or receptors, are engendered by, and act only against, the proteins present in the bodies (soma) of the bacilli. These body proteins, or *somatic proteins*, are usually not species-specific but may occur in several species of any group of closely related organisms as previously described. Such agglutinins, being common to a group of organisms, are often called *group agglutinins*.

**Receptor Analysis.**—The H or flagellar proteins of motile bacilli may be destroyed by treatment of the bacteria with warm alcohol (37° C.). Thus, we may obtain a suspension which will react only to the O antibodies of a given serum although the serum may also contain H agglutinins. (A nonmotile variant will react in the same way.) The O proteins are not so readily affected by heat, but we may prepare a suspension of flagellated organisms which will not react to O antibodies, but only to H, by treatment with formalin or

\* In this discussion the student must bear in mind *constantly* the distinction between *antigen* and *antibody*.

phenol. The flagella are stiffened and protrude from the bacilli like spines on a sea-urchin. Thus the flagella can come into contact and form *large flocculent* clumps under the influence of the H agglutinins, while the bodies of the bacilli are held apart although O agglutinins may be present. When the O agglutinins act on O antigens without the interference of flagella, *very fine granular* clumping is observed.\* Such preparations are of great use in diagnosis as they enable us to analyze a patient's serum with respect to the types of receptors (or agglutinins) present. The process is called *receptor analysis*, and is useful in studying disease.

For example, the serum of persons recently *infected* with an organism of the typhoid-paratyphoid-dysentery group will show a characteristic marked *increase*† in the amount of somatic receptors or O agglutinins for this group in his serum. Thus, we may determine to what group the infecting organism belongs. If there is also marked increase in the concentration of H agglutinins, it is possible to state as a rule, but not with absolute certainty, which *species* in the group is causing the disease, since the H agglutinins are, with few exceptions, species-specific.

A person *injected* with a vaccine made of killed bacteria of the group, as is often done for preventive purposes (*vide infra*), after the lapse of a few weeks or months will usually show little or no *permanent* increase in O agglutinins but a persistent increase in H agglutinins corresponding in type to the species of bacteria injected. Thus, we may often determine by receptor analysis test not only the species of organism causing the appearance of the agglutinins but also whether a person's agglutination reaction is due to actual infection at the time of the test or merely to a previous *prophylactic* (protective or preventive of disease) injection.

**Precipitins.**—In the agglutination reaction whole cells, like bacilli or erythrocytes or other cells, are clumped together. *Pre-*

\* **Antigen for demonstrating H agglutinins:** With as little agitation as possible, wash the growth of a motile strain of *Eberthella typhosa* from a 24-hour-old agar slant culture with 0.85 percent NaCl solution and dilute to an appropriate density. Add 0.2 percent formalin and store for use. In testing, incubate the serum-antigen mixture at 37° C. for 2 hours.

**Antigen for demonstrating O agglutinins:** Proceed as above, using a minimum of saline solution. Add equal volume of absolute alcohol, mix and store for 24 hours at 37° C. Shake once or twice. Add sufficient saline solution to bring alcohol to 33 percent concentration (1/2 total volume). Store in the cold. Keeps well. Dilute with at least 5 volumes of saline solution before use. In testing, incubate the serum-antigen mixture at 50° C. for 24 hours.

† Normal serum often contains small amounts of certain antibodies, especially agglutinins of various sorts.

*cipitins*, which seem to be very closely related to agglutinins and which are probably merely a different manifestation of the same antibodies, cause the clumping and precipitation of *invisible molecules* of protein so that a visible turbidity or flakiness is formed as described in the section on toxin and antitoxin (see page 297). Serological identification of soluble proteins of closely similar composition is thus possible, a scientific feat impossible of attainment by the chemist. The procedure is similar to that used in the agglutina-

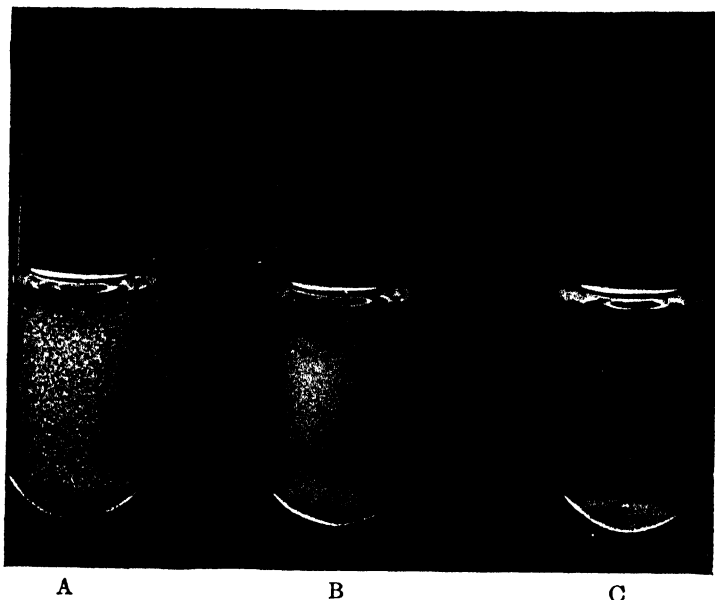


Fig. 145.—The precipitin test in syphilis. *A*, Strongly positive. Definitely visible particles are suspended in the transparent medium. *B*, Weaker reaction. Fine particles are suspended in a somewhat turbid medium. *C*, Negative reaction. The medium is transparent and free from particles. (From Kahn, "The Kahn Test," Williams & Wilkins Co., publishers.)

tion test. It is very simple and is valuable in industry, chemistry and legal medicine. For example, it is possible to determine whether minute blood stains are of human or some other origin, even when the stains are many years old. This test is a favorite with some writers or scientific detective novels. Pure proteins derived from bacteria and other sources can also be studied in the same way.

*The Precipitin Test Applied to Syphilis.*—It seems to be well established that, in complement fixation tests such as the Wasser-

mann reaction, the complement is fixed because it is *adsorbed* onto finely divided particles of precipitate resulting from an interaction between the antigen and antibody. Unfortunately, in the Wassermann reaction, the precipitate formed is not visible. It would be a very great advantage if, instead of having to test for the presence of this invisible precipitate by adding complement and then being forced, in turn, to test for the presence of complement by adding

## STEPS IN SLIDE TEST

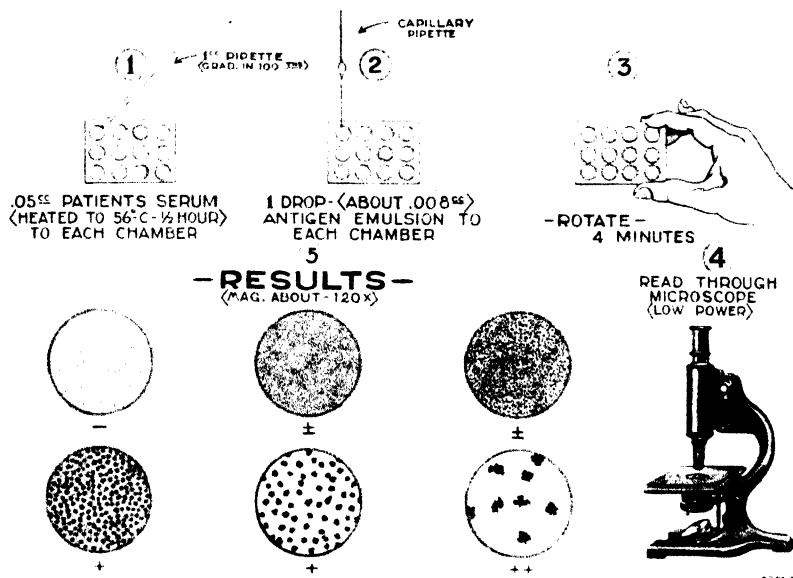


Fig. 146.—Equipment and procedure in the Kline slide precipitation test. (Courtesy of Dr. B. S. Kline.)

sensitized red corpuscles, we could see the precipitate directly as in other precipitin reactions. This has been accomplished.

A specially prepared and very concentrated alcoholic antigen is used, in which the reactive substances are present in the form of large, unstable, colloidal complexes. These are brought, by proper dilution with saline solution, to a state where, in contact with syphilitic serum, they precipitate in a visible form while no precipitation occurs in the presence of normal serum (Fig. 145).

*The "Slide Reaction."*—In the last few years the "slide reaction"

has gained great favor as a rapid routine method in performing the agglutination or precipitin test. In this method a loopful or a tenth of a cubic centimeter of the serum to be used, properly diluted if necessary, is placed on a glass slide. The antigen, either soluble or bacterial suspension, is immediately added by means of a loop or a measuring pipette and the serum and antigen are gently mixed with a loop or by tilting the slide back and forth. Very often the reaction is discernible with the naked eye as a granular or "curdled" appearance of the antigen; other times it must be observed with the high-dry lens of the microscope (Fig. 146). The method is widely applicable for soluble protein antigens (precipitin tests) and for cellular antigens (agglutination tests). The reaction shown in the figure follows the procedure devised by Kline for the diagnosis of syphilis. Glass plates with depressions to hold the serum-antigen mixtures are often used, as in the Eagle test for syphilis.<sup>10, 11, 12</sup> In a simple procedure devised by Powell,<sup>13</sup> a drop of the desired killed antigen, previously mixed with just enough methylene blue to give a definite color, is placed on hard-surfaced paper board or good bond paper and spread with a needle over an area about 1 cm. in diameter. A drop of the serum is added immediately and mixed with the antigen. Flocculation occurs in a few minutes, and when dried the preparation serves as a permanent record.

**The "Halo" Reaction.**—Another method of demonstrating the precipitin reaction is to incorporate the antiserum in nutrient agar, pour the latter into a Petri dish, and inoculate the surface with species of bacteria to be tested. As growth occurs, soluble precipitable substances diffuse from the growth into the agar where they react with the serum, causing a smoky or milky halo to appear (Fig. 147).<sup>14</sup>

The method is available with meningococci, and pneumococci and other species producing a soluble specific substance (S.S.S.). Fresh pneumonic sputum, containing much S.S.S., gives a similar reaction and the method can be used for bedside type determination. In this case, type-specific sera are mixed with agar in small precipitin tubes and the sputum layered on the agar.

**Protective Antibodies.**—All of the immune reactions so far mentioned have been demonstrable by test tube methods. It was mentioned that *immunity* does not necessarily result from the presence of such antibodies. Indeed, the exact significance of these antibodies for protection against disease is not always clear at all. When this became known scientists sought for a more exact measure of the power of the serum to protect against infection. What better

method could be devised than actually to infect experimental animals (*e.g.*, mice) and give them doses of the serum to be tested to see whether they are thereby protected? This measures *protective power* directly, regardless of whether this power depends on agglutinins, cytolsins, or some still undiscovered antibody.

Such a test is known as a *protection test* and is widely used to measure the antigenic virtue of antigens and the protective power of sera. For example, suppose we have a typhoid bacillus suspen-

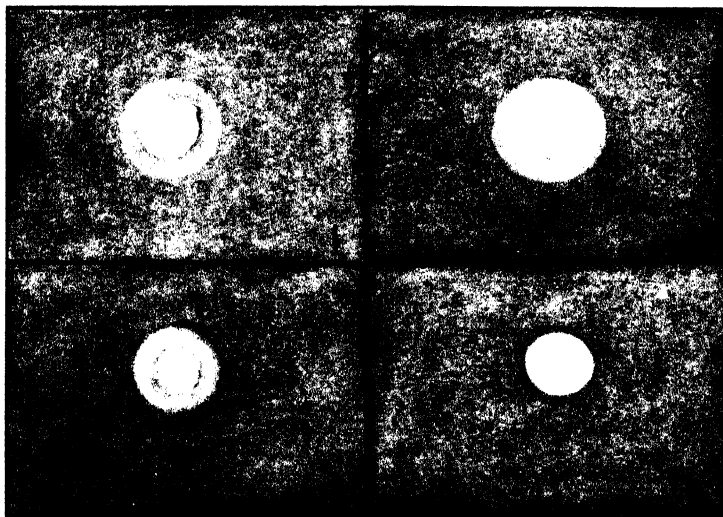


Fig. 147.—A drawing of the precipitate around colonies of group I-III meningococcus on immune serum agar plates after 72 hours of incubation. (Pittman, Branham and Sockrider, U.S.P.H.R., Vol. 53.)

1. Plate contained 0.1 cc. of immune serum. Halo intensity +.
2. Plate contained 0.2 cc. of immune serum. Halo intensity ++.
3. Plate contained 0.5 cc. of immune serum. Halo intensity +++.
4. Plate contained 1.0 cc. of immune serum. Halo intensity ++++.

sion which we wish to test for antigenicity. Suppose it is to be used for inoculating soldiers against typhoid fever during maneuvers. Our best procedure is to inoculate several persons and then test their serum for antibodies. But shall we test for H agglutinins, or O agglutinins, or some other? We can best find out if the serum of such persons *actually protects* against typhoid infection by injecting a small amount (say 0.5 cc.) into a mouse and shortly afterward giving him an injection of live typhoid bacilli just about large



enough to kill were no protection given by the serum. By varying the dosages of serum and bacilli, one may measure the *protective potency* of the serum. This method is, in fact, actually in use by the United States forces at present and represents one of the many great steps forward in the application of scientific discoveries to military procedure. It was adopted after very extensive investigations by Siler, Callender, Longfellow, Luippold and their colleagues in the Army Medical School at Washington, D. C.<sup>15</sup>

The protection test is of course useful in many other applications. A similar test, often called the *neutralization test*, is used in connection with filtrable viruses (see section on viruses, page 726).

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## CHAPTER 17

# RELATIONS OF IMMUNOLOGY TO BACTERIOLOGY

## II. PRODUCTION OF IMMUNITY AGAINST INFECTIONS

IN THE LAST CHAPTER we discussed antigens and antibodies in general, and the kinds of reactions that occur between them, as well as methods of studying immune reactions *in vivo* and *in vitro*. In that chapter it was pointed out that persons might become immune to certain diseases by surviving natural attacks of those maladies. The body acquires specific properties of resistance against such infections. Examples cited were diphtheria and typhoid fever. This sort of immunity, not existent in the normal person, is acquired in the course of natural events and is therefore spoken of as *naturally acquired immunity*. In this chapter we shall speak of some other kinds of immunity, including *nonspecific* and *species* immunity, but more especially of purposefully induced immunity, or *artificial immunity*.

**Nonspecific Immunity.**—The term *nonspecific immunity* (or, better, *nonspecific resistance*) is sometimes applied to a rather varia-

ble, general resistance to disease, depending on robust health and vigor. For example, a person much weakened by the development of cancer, tuberculosis, starvation or diabetes usually has poor resistance, or little nonspecific immunity, to infection in general.

**Species or Racial Immunity.**—There are certain diseases to which certain species seem never to be subject. For example, horses never have measles, men never have fowl plague, fish never have typhoid fever and human beings never have "Texas fever" of cattle. Certain Oriental and Semitic peoples are less susceptible to tuberculosis than Caucasians. Certain kinds of rabbits never have "snuffles." This natural, inherited resistance is called *species* or *racial immunity*.

**Artificial Immunity.**—As outlined above, immunity to disease may result naturally from racial, species or individual characteristics, or from actual infection or contact with infective agents. In the latter case, although mild or subclinical\* infections often occur, with the development of immunity as a result, diseases causing immunity are all too frequently very severe and may be disabling and disfiguring or fatal. We have no entirely reliable control over natural disease.

It would be much better if we could become immune by some means which we can direct and so avoid the dangers and discomfort necessarily inherent in the natural process. Furthermore, we should like to become surely and safely immune to disease early in life and not have to wait for accidental natural infection, occurring perhaps at a very inconvenient time in adult life. In addition, it is often desirable to be able to produce an immunity to certain diseases at certain definite times. For example, a person desiring to do laboratory research with yellow fever virus would like to be able to immunize himself safely and surely before starting the work, since natural infection with the virus is very apt to result fatally. So also, physicians and nurses or others working with typhoid or diphtheria patients should be immunized safely and comfortably against these diseases in time to begin their work.<sup>1</sup> All this, however, is too much to expect of Nature.

In view of these needs, man, following the lead of Pasteur, has devised means of developing specific immunities "artificially" and safely. The methods involve natural processes, but are used under *modified and carefully controlled* conditions and are therefore called

\* Subclinical infections are those in which symptoms are so very mild that no special attention is paid to them. The vast majority of infections, fortunately, are of this type.

"artificial immunization." Two types of artificial immunity are used: *active artificial immunity* and *passive artificial immunity*.

**Active Artificial Immunity.**—In active artificial immunity the patient's body is stimulated to produce antibodies by being injected with certain kinds of antigens. These are of three general types, as follows: (a) sterile bacterial exotoxins, or (b) sterile bacterial cellular or somatic antigens (proteins and carbohydrates), or endotoxins, in the form of dead bacteria, or (c) living, infectious bacteria, the virulence of which has been reduced or *attenuated* by various procedures so that no *serious* infection results.

**Immunization with Toxins.**—The first (a) is the simplest procedure and will be readily understood by those who have read the preceding chapter. Cultures of toxin-producing bacteria, like *C. diphtheriae*, are made in broth and, after sufficient growth, are passed through porcelain filters which remove the bacteria. The *filtrate* (broth passing through the filter) contains the exotoxin. This may be injected hypodermically (under the skin) in from 1 to 5 very minute doses at weekly intervals into the persons to be immunized and eventually (usually after two to six weeks) their blood will be found to contain antitoxin which protects them from the toxin in question. This procedure or a modification of it was formerly used in immunizing children against diphtheria. There is considerable danger, however, and many fatal accidents occurred due to overdoses of toxin even when a little antitoxin was mixed with it.

**Toxoids.**—Soon after its discovery it was observed that diphtheria toxin is quite unstable and rather rapidly undergoes spontaneous deterioration, especially if exposed to a warm climate or sunlight. The toxin is altered by warmth and light, and also by such substances as formaldehyde, until it is no longer poisonous. It is then spoken of as *toxoid*. Far from losing its value in such a condition, it has been found that toxoid, in spite of being nontoxic, nevertheless retains its specific antigenicity and engenders antibodies which neutralize the toxin. In order to avoid the dangers attendant upon the use of toxin, toxoid came into general use for active immunization against diphtheria.

It was later found possible to improve such antigens still further. The addition of alum to broth containing the toxoid *precipitates* the latter and it may then be collected, concentrated and purified to some extent. Alum-precipitated toxoid is highly effective and only one injection is needed as compared with two or three injections of fluid toxoid antigen. Similar discoveries have been made concerning

other toxins used in active artificial immunization, notably the toxins of scarlet fever and tetanus. Kendrick and Eldering<sup>18</sup> have adapted the principle to bacterial vaccine against whooping cough (see page 637).

*Action of Adsorbed Toxoids.*—It is important to understand why alum-precipitated toxoid is more effective than plain, fluid toxoid. When the latter is injected under the skin, the soluble material and fluid are quickly adsorbed by the body and destroyed and eliminated inside of a few hours. The *antigenic stimulus* is very transitory, and repeated injections are necessary. If the toxoid could be held *in situ* for several days, being released continuously little by little into the body, the antigenic stimulus would be prolonged and continuous and there would be no need for further injections.

Actually, this is accomplished by means of the alum-toxoid. The toxoid is firmly adsorbed and held on the surfaces of the alum precipitate, which is insoluble. The alum precipitate remains *in situ* undissolved, releasing its *adsorbed* toxoid slowly, little by little, giving the patient a prolonged and continuous antigenic action which is highly effective.

This principle is not confined to diphtheria immunization but is of broad fundamental significance. The existence in the body of an antigen, living or dead, which persists there and maintains immunity by its continuous antigenic action, is sometimes spoken of as "premunity."

**Primary and Secondary Stimulus.**—Another important fundamental principle is illustrated by the effect of repeated doses of antigen. Suppose that a child be given a single dose (1 cc.) of alum toxoid. In two weeks, his blood, tested by appropriate methods, shows very few antibodies. After four weeks, however, his blood is found to contain a satisfactory amount of antitoxin (say 0.5 unit per cc.). The development of immunity has been slow! A year later this is found to have diminished to a very low figure (say 0.001 unit). This diminution of antibody concentration in the serum is very common in all types of immunization. As previously mentioned, it does not necessarily mean that there is a corresponding diminution in resistance to the disease. It may mean that antibodies remain attached to the tissue cells instead of circulating in the blood.

After time has reduced the effect of the first antigenic stimulus, let us give the child a very minute *second* dose of toxoid (say 0.005 cc.) and test his blood for antitoxin at short intervals. A surprisingly rapid and extensive response is noted. Whereas, after the *first*

or *primary stimulus* given a year before, response was slow (even to a large dose of toxoid; 1 cc.), response now to  $\frac{1}{200}$  this dose, when given as a *secondary stimulus*, occurs in a few hours and the child may be found to have 1 or more units of antitoxin per cc. of blood inside of two or three days (Fig. 148).

It is as though the body cells, having once had an "antigenic experience," are more "expert" at forming antibodies of this particular sort and do so with great facility whenever called upon. It is upon this very rapid reactivity that the child's ability to withstand the disease may largely depend. It explains, in part, why resistance to disease and permanent concentration of demonstrable

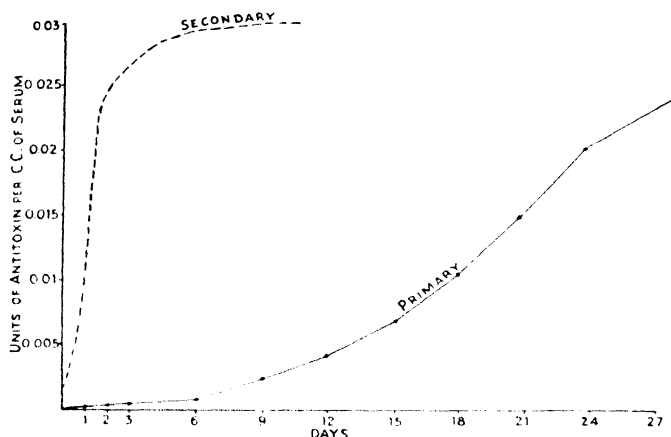


Fig. 148.—Curves showing rate of antitoxin production following a primary injection and following a secondary injection. Note that after the secondary injection antitoxin production is much more rapid and extensive than after the first or primary stimulus.

antibodies in the serum are not necessarily related. Each time the specific antigen comes into contact with the child's tissues, the latter react like a well-trained "flying squad" of guardsmen and repel the invader before it can gain any foothold and cause disease.<sup>2, 3</sup> The principle of the primary and secondary stimulus is a generally applicable one and should be borne in mind. It is not restricted to toxins but works equally well with living or dead bacteria, egg white, and other substances.

**Immunization with Dead Bacteria.**—The second procedure of active, artificial immunization is very similar to the foregoing except that, instead of removing bacteria from the broth in which they have grown and using their excreted growth products, the bacteria

themselves are used. For convenience, greater yield, and avoidance of foreign matter, they are usually cultivated on agar and removed to physiological saline solution which is then heated to 60° or 70° C. to kill the bacteria. A very minute amount (0.25 percent) of phenol or tricresol is added to insure sterility.

Such suspensions of killed bacteria are frequently referred to as vaccines but they are correctly termed *bacterins*; the term "vaccine" is properly restricted solely to the immunizing agent against smallpox.

Methods of killing bacteria and viruses for vaccines by means of ultraviolet irradiations seem to give good results.<sup>3a</sup>

The method of employing bacterins is so effective in preventing typhoid and paratyphoid fever that their use against these diseases in the United States military forces has become a matter of regulation. The principle of the secondary stimulus is now made use of by the U. S. Army in connection with these bacterins. After a soldier has received an ordinary course of three weekly inoculations as a primary stimulus, his resistance is maintained by single, annual, intradermal injections of 0.1 cc. of bacterin. These cause little or no reaction, are quick, inexpensive and easy and, above all, effective in reinforcing waning immunity.

The hypodermic injection of carefully controlled doses of suspensions of killed staphylococci is also much used to increase resistance of persons susceptible to boils, styes, etc., which are usually due to staphylococci. The use of *Hemophilus pertussis* bacterins in preventing whooping cough is of great value also. Kendrick and Eldering have adapted the method of alum precipitation, used for toxoids, to these bacterins with great success.

**Antigenic Strains of Bacteria.**—An important principle in the use of bacterins has been demonstrated especially in connection with typhoid and pertussis immunization. We have noted that bacteria, when invading the body, often undergo variations which increase their virulence. Such changes may involve the acquisition of capsules or certain obscure alterations in physical or chemical make-up which aid them in invading the body. Upon isolation from the body and cultivation on artificial media, they lose these peculiar properties and revert to a more or less saprophytic state. In this state, while still capable of infecting under favorable circumstances, they are much less irritating or virulent (or antigenic) and may, indeed, prove quite ineffective as bacterins. It is important, therefore, to select, for bacterins, freshly isolated organisms of known antigenic value.

In the case of *Hemophilus pertussis* the organisms when freshly isolated from a pertussis patient and in a properly *antigenic* state are of the smooth, encapsulated type and are highly virulent for mice. This condition of the organisms is spoken of as "phase I." In the case of *Eberthella typhosa*, the antigenic strains are smooth, highly virulent for mice and exhibit a certain component called the "Vi antigen" in enhanced degree. Such strains stimulate production of *protective antibodies* which may be distinct from agglutinins, etc. Organisms devoid of these special properties may stimulate the production of certain antibodies, as agglutinins or cytotoxins, but this does not always mean that they produce effective immunity or protective antibodies to disease since, as has been pointed out, immunity and test-tube-demonstrable antibodies are not synonymous.

**Immunization with Attenuated, Living, Infectious Agents.**—The third method of artificial active immunization consists in actually infecting the person to be immunized, with the desired organism which is of greatly attenuated or modified virulence. It is of relatively little importance in artificial production of immunity to bacterial disease but is probably the most common and effective *natural* method in bacterial infections. People are continually coming into contact with living infectious agents, bacterial and otherwise, yet the vast majority of these contacts never result in severe disease but often in subclinical infections, suggesting that common strains of infectious bacteria may have attenuated virulence. This attenuated-virulence concept, however, is not proven and is a very knotty question indeed. It may be that resistance of people is usually high.

There are at least three means of lowering the virulence of pathogenic organisms so that they can be safely used to induce active, artificial immunity. These are *animal passage*, *desiccation*, and *cultivation on special media* or *under special conditions* such as abnormally high temperature.

*Animal Passage.*—Possibly the only purely *bacteriological* use of the principle was tested by Pasteur who attenuated the organisms of swine erysipelas (*Erysipelothrix rhusiopathiae*) by injecting them into rabbits and then recovering them and treating swine with them. The swine often became immune after a usually mild attack of the disease. This method is not used extensively in the United States.

Another form of this process is very well illustrated by the method of preparing material for the classical vaccination against



smallpox, although the organisms causing this disease are not bacteria. They belong to the group of living, pathogenic agents called *viruses*, which are too small to be seen with the ordinary microscope



Fig. 149.—Cowpox. Showing well advanced lesions on the teats and udder (Courtesy of Robert Graham from Hagan, "The Infectious Diseases of Domestic Animals," Comstock Publishing Co., Inc.)



Fig. 150.—Edward Jenner, 1749–1823. (From the painting by Sir Thomas Lawrence.)

and which pass filters capable of holding back ordinary bacteria (see chapter on Viruses, page 703). However, the principle involved is the same whether viruses or bacteria are used.

Smallpox virus causes *smallpox* in human beings. It can also

infect cows and horses. The disease in cows, however, is quite mild and never results fatally, and the pustules remain localized, usually on the cow's teats and udder (Fig. 149). This latter disease is called cowpox or *vaccinia* (*vacca* = cow). Cowpox can be transmitted to human beings but is never the same as the original smallpox. The smallpox virus appears to become modified in some unknown manner by contact with the cow, so that it causes only a very mild harmless infection of human beings.

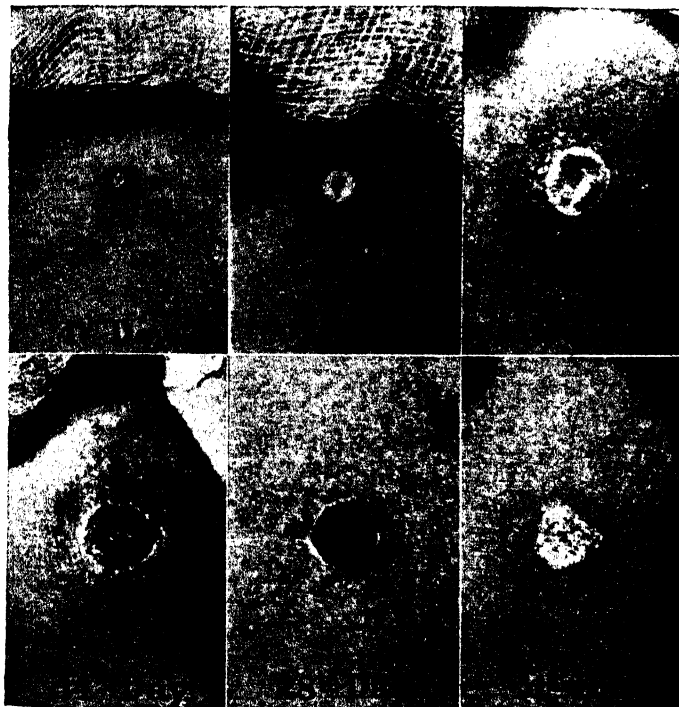


Fig. 151.—The course of the eruption in Jennerian vaccination. (From Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

In 1798 Jenner (Fig. 150), then a country doctor but later a very famous British scientist, observed that many dairy workers associated with cows having cowpox did not succumb during epidemics of smallpox. Experimenting, he found that if some of the serum or lymph from the pustules on the udder of a cow with cowpox were scratched into the arm of a human being, a very mild disease (*vaccinia*) resulted, with the formation of a single, localized, poxlike

lesion which soon healed, leaving a distinctive scar (Fig. 151). *Smallpox* never developed in persons *after* infection with *cowpox*. The person thus safely became immune to smallpox through infection with cowpox, cowpox virus being, as we have said, really smallpox virus in a modified form.

Vaccine virus, as used today, is prepared by scratching cowpox or smallpox virus into the shaved and disinfected skin of a calf. When the pustules are "ripe" the lymph is collected from them and put up in glass tubes ready for use (Fig. 152).



Fig. 152.—Collecting of cowpox lymph from the skin of a calf. The skin has been shaved, cleaned, disinfected and inoculated in long parallel scratches (clearly seen in the picture) with cowpox lymph. Typical pustules have developed along the scratches and the lymph from these is being collected with surgical cleanliness. (From Monteiro and Godinho.)

Its potency and cleanliness are carefully controlled by the National Institute of Health at Washington, D. C. Its value is made evident by experience and by a glance at Figure 153. However, it is never free from numerous contaminating bacteria and is very perishable.

Rivers and his coworkers at the Rockefeller Institute<sup>4</sup> devised means of cultivating vaccinia virus in living cells of the chick, suspended in a nutrient fluid (Tyrode's solution; see page 162), so that

it is now possible to use vaccine which contains no bovine substance whatever. Buddingh<sup>6</sup> and his colleagues developed a method for producing the vaccine in living chick embryos. Such vaccines have the further enormous advantage of being free from all bacteria, especially the germs of tetanus (lockjaw) which sometimes (but very rarely) cause trouble in the older method. Furthermore, as the cultivated virus is injected into the skin there is no ulceration and consequently no disfiguring scar. Also, culture virus may be kept in



Fig. 153.—Children of one family who were brought to the Municipal Hospital of Philadelphia with the mother and father, who had smallpox. The child in the center had been considered too young to be vaccinated. The other children had been vaccinated a year before; they remained free from the disease, although for several weeks they lived in the wards of patients with smallpox. (From Welch and Schamberg, "Contagious Diseases," Lea and Febiger, publishers.)

a dried state for long periods without deterioration. The older method of Jennerian vaccination is still most widely used.

**Desiccation.**—*Immunization Against Rabies.*—Pasteur's name is immortalized in the term "Pasteur treatment" for *rabies* or *hydrophobia* ("mad-dog bite"). The process is not really a curative "treatment" but is a course of immunizing injections with the living but attenuated virus of rabies. If started soon enough after the bite of the dog, immunity develops from the injections before the virus from the dog-bite can cause disease.

For years Pasteur had experimented with rabbits, dogs and

guinea pigs (one of the many brilliant illustrations of the value of animal experimentation) until he felt morally certain that his method of attenuating rabies virus was safe and effective. It consisted, not in passing the virus from cow to man as in smallpox, but in first passing it from rabbit to rabbit to establish a uniformity of infectivity and an *attenuated* virulence for man. Strangely, it has a *maximum* and fixed virulence for rabbits, and Pasteur called this virus "virus fixé." The potency of the virus is further reduced

by drying (Fig. 154) or dilution (Högyes method) and is injected in increasing doses.

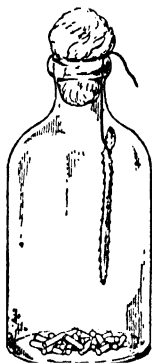


Fig. 154.—Method of drying the spinal cord of a rabbit dead of rabies, for the purpose of attenuating the virus. The cord is removed from the animal with aseptic technique, and suspended by a sterile thread in a bottle over pieces of potassium hydroxide. This substance has a great power of absorbing moisture. (From Hiss, Zinsser and Russell, "Text-Book of Bacteriology," D. Appleton-Century Company, publishers.)

Pasteur's first human immunization against rabies, in 1885, was an extremely dramatic event and marked an epoch in the progress of the war on disease. Indeed, Pasteur is referred to as "the father of immunology." His experiments on rabies have been well described by Vallery-Radot, Pasteur's grandson. The patient was a little Alsatian boy named Joseph Meister. Pasteur consulted his friends, Vulpian and Grancher.

"Vulpian and M. Grancher examined little Meister, and seeing the number of bites, some of which, on one hand especially, were very deep, they decided on performing the first inoculation immediately." The substance chosen was the dried spinal cord of a rabid rabbit. It was fourteen days old and had quite lost its virulence. It was to be followed by further inoculations, gradually increasing in strength. As the process of immunization continued, the potency or dose of the rabies virus used was gradually increased. More and more the danger from actual infection of the boy by the dreadful virus grew. Pasteur, although confident, was nevertheless watching with great care the result of his injections.

"'All is going well,' Pasteur wrote to his son-in-law on July 11th; 'the child sleeps well, has a good appetite, and the inoculated matter is absorbed into the system from one day to another without

leaving a trace. It is true that I have not as yet come to the test inoculations, which will take place on Tuesday, Wednesday and Thursday.' . . . But, as the inoculations were becoming more virulent, Pasteur became a prey to anxiety: 'My dear children,' wrote Mme. Pasteur, 'your father has had another bad night; he is dreading the last inoculations on the child. And yet there can be no drawing back now! The boy continues in perfect health.' " The injections continued. Pasteur's hopes rose, as shown in the following letter to Mme. Pasteur:

" 'My dear Rene, I think great things are coming to pass. Joseph Meister has just left the laboratory. The three last inoculations have left some pink marks under the skin, gradually widening and not at all tender. There is some action, which is becoming more intense as we approach the final inoculation which will take place on Thursday, July 16. The lad is very well this morning, and has slept well, though slightly restless; he has a good appetite and no feverishness. He had a slight hysterical attack yesterday.'

"The treatment lasted ten days; Meister was inoculated twelve times. The virulence of the medulla (spinal cord) used was tested by trephinations [intracerebral inoculations] on rabbits, and proved to be gradually stronger. Pasteur even inoculated, on July 16, at 11 A. M., some medulla only one day old, bound to give hydrophobia to rabbits after only seven days' incubation; it was the surest test of the immunity and preservation due to the treatment." Nothing untoward occurred.

"The treatment being now completed, Pasteur left little Meister to the care of Dr. Grancher (the lad was not to return to Alsace until July 27) and consented to take a few days' rest. He spent them with his daughter in a quiet, almost deserted country place in Burgundy, but without however finding much restfulness in the beautiful peaceful scenery; he lived in constant expectation of Dr. Grancher's daily telegram or letter containing news of Joseph Meister."

The boy never developed rabies, however, and returned home quite well. A new method of immunization had been demonstrated.

Today immunization of dogs against rabies is accomplished by means of nervous tissues containing virus inactivated by various means. In the Semple method it is treated with phenol. Kelser and Schoening,<sup>6</sup> and Leach<sup>7</sup> have shown the value of chloroform-treated vaccine. Webster<sup>8</sup> and his coworkers have demonstrated that virus inactivated by ultraviolet light has considerable value. The great difficulty with preparations in which the virus is killed is that so

little actual virus is present that the antigenic stimulus is small. Very large doses have to be given. To overcome this Kligler, et al.,<sup>9</sup> have cultivated the virus in the brains of chick embryos where very large quantities of virus grow. Such material, inactivated with formaldehyde, has given good results in preliminary tests on dogs.

**Cultivation in Special Media.**—*Immunization Against Tuberculosis.*—A method for immunization against tuberculosis has come into prominence. The procedure is known as the "Calmette process" and the attenuated culture as "B. C. G." (*Bacillus Calmette-Guérin*). The value of the method is still in question, but the data available seem promising. The process consists of a series of injections of *living* tubercle bacilli, the virulence of which has been reduced by cultivation of the organism on certain media containing bile. This procedure probably induces the development of a stable variant of low virulence. Similar variants may easily be produced in other culture media. This is probably the only important use to date for immunization of human beings with bacteria, attenuated by cultivation on special culture media.

**Attenuation by Cultivation at Unfavorable Temperatures.**—Although this method is not much used at present, its discovery and demonstration by Pasteur constitute an episode of great dramatic interest in the history of bacteriology. The principle was first used with *Pasteurella avicida*, the cause of fowl cholera. It was later developed in connection with studies of anthrax which, in the nineteenth century, decimated the sheep flocks of France and still does so in some parts of Europe. Following numerous preliminary experiments by the tireless Pasteur, on variants of *Bacillus anthracis*, cultures of the organisms were prepared by cultivation at temperatures of from 39° to 43° C. (optimum around 35° C.) which caused them to lose some of their virulence (and also, often, their power of spore formation).\* The immunizing agent finally adopted by Pasteur consisted of broth cultures of these attenuated strains. The first great public demonstration of the value of anthrax immunization gave the indomitable Pasteur some uncomfortable hours, because his ideas had been very unfavorably regarded by many and his experiments were very closely watched. He injected a certain number of sheep with his attenuated organisms. After a suitable period had been allowed for antibodies to develop, these animals, as well as an equal number of normal, unprotected ani-

\* The method has since been shown to vary in result so that neither loss of spores nor cultivation at 42° C. should be assumed to have deprived these organisms of their dangerous properties.

mals, were to be publicly inoculated with large doses of fully virulent anthrax bacilli. This experiment has been dramatized in a motion picture. The test injections were made on May 31, 1881, at Pouilly le Fort. There were many skeptics in the audience when the injecting began.

"At half-past three [on May 31, 1881] everything was done [*i.e.*, the test injections of protected and unprotected sheep completed], and a rendezvous fixed for June second at the same place. The proportion between believers and unbelievers was changing. Pasteur seemed so sure of his ground that many were saying, 'He can surely not be mistaken.' One little group had that very morning drunk to a fiasco. But, whether from a sly desire to witness a failure, or from a generous wish to be present at the great scientific victory, every man impatiently counted the hours of the two following days.

"On June fourth, Messrs. Chamberland and Roux [Pasteur's assistants, and later famous scientists] went back to Pouilly le Fort to judge of the condition of the patients. Amongst the lot of unvaccinated sheep, several were standing apart with drooping heads, refusing their food. A few of the vaccinated subjects showed an increase of temperature; one of them even had 40° C. (104° F.); one sheep presented a slight edema of which the point of inoculation was the centre. One lamb was lame, another manifestly feverish, but all, save one, had preserved their appetite. All the unvaccinated sheep were getting worse and worse. 'In all of them,' noted M. Rossignol, 'breathlessness is at its maximum; the heaving of the sides is now and then interrupted by groans. If the most sick are forced to get up and walk, it is with great difficulty that they advance a few steps, their limbs being so weak and vacillating.' Three had died by the time M. Rossignol left Pouilly le Fort. 'Everything leads me to believe,' he wrote, 'that a great number of sheep will succumb during the night.'

" 'This morning, at eight o'clock,' wrote Mme. Pasteur to her daughter, 'we were still very much excited and awaiting the telegram which might announce some disaster. Your father would not let his mind be distracted from his anxiety. At nine o'clock the laboratory was informed, and the telegram handed to me five minutes later. I had a moment's emotion, which made me pass through all the colours of the rainbow. Yesterday, a considerable rise of temperature had been noticed with terror in one of the sheep; this morning that same sheep was well again.' "

A day or two later, when Pasteur arrived at Pouilly le Fort, "the carcasses of twenty-two unvaccinated sheep were lying side by



side; two others were breathing their last; the last survivors of the sacrificed lot showed all the characteristic symptoms of splenic fever. All the vaccinated sheep were in perfect health.”\*

No success had ever been greater than Pasteur's (Fig. 155). The most incredulous were now convinced, and desired to become



Fig. 155.—Pasteur's Jubilee 1892. The painting represents Lister greeting Pasteur at the Sorbonne.

the apostles of his doctrine. M. Biot spoke of nothing less than of being himself vaccinated and afterward inoculated with the most active bacteria.

\* From "The Life of Louis Pasteur," by René Valléry-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

**Passive Immunity.**—An *important point* to remember in connection with all active immunity, whether due to natural disease or to the artificial use of antigens, is that when antigens first gain entrance to the body a period of time, possibly two to six weeks, may elapse before the antibody response is evident. This interval is often called the *latent period*. If the antigen gaining entrance to the body be diphtheria toxin from diphtheria bacilli infecting the throat, this lapse of time is of great importance if the patient is suffering from a severe, clinical case of the disease. Obviously treatment with bacterins, toxoids or attenuated organisms would prove futile. The toxin is formed by the bacteria so very rapidly that the



Fig. 156.—Commercial syringe—package of antitoxin.

patient may be overwhelmed and die before his body cells can possibly manufacture enough antitoxin to combat the invader. A single hour may mean the difference between life and death.

What an advantage it would be if the desperately needed antibodies could be immediately supplied in generous excess from some outside source to tide the patient over his emergency! These antibodies are now available in large quantities in the form of serum taken from a previously immunized animal. Such antibody-containing serum can be purchased at any well-stocked drug store in sterile syringes all ready for injection (Fig. 156). It is like a loan of life from the great reserve bank of nature to avert an immunological

overdraft! Unfortunately not all infections are amenable to serum therapy. For example, good serum is not available for the treatment of staphylococcus infections or tuberculosis. Such organisms seem to have little power to engender protective antibodies in such amount that the serum has value in passive immunity. They are said to be poor antigens.

**Commercial Manufacture of Antitoxin.**—Antitoxin-containing serum, be it diphtheria or tetanus antitoxin, or any other sort, is taken from strong, clean and healthy horses or other animals which have previously received a long series of injections of the proper antigen. The first doses of antigen injected into the horses are



Fig. 157.—The production of diphtheria antitoxin from horses. (Reproduced from Therapeutic Notes, by courtesy of Parke, Davis & Company.)

small, but increase in size as the animal becomes more and more immune and his blood contains more and more antibody. At the end of several months a quantity of blood is drawn from the animal and the serum removed after the formation and contraction of the clot (Fig. 157). The serum contains the antibodies in large amounts. Various methods of extracting and concentrating the antibodies are used so that the human patient may receive large amounts of antibody in as small a dose of serum as possible.

**Transitory Nature of Passive Immunity.**—When patients receive injections of such antibody-containing serum, they become *immediately* immune, not through any active antibody formation on their own part, but by the *passive reception* of ready-formed antibodies.



respect to the antigen involved. The immune state and the allergic state, while closely related, are not identical and one may exist in the absence of the other. The entrance of an antigen into the body parenterally, regardless of source or nature, be it bacteria, toxin, serum, milk, oyster, plant juice or what you will, not only engenders circulating antibodies against that antigen, a benign reaction, but also alters the body tissues so that a second injection of the same antigen, given *about two weeks* after the first, produces an immunological response which is much more rapid and vigorous than the first. This response may be perceptible only as an increase in antibody production, or it may manifest itself in very striking, often distressing, ways.

It may be supposed that the tissues contain antibodies fixed to the cells which produced them. Then an antigen-antibody reaction takes place immediately on or in those cells, with varying results, the nature of which depends on the species of animal, the kind and location of the cells, the violence and extent of the reaction, and whether or not the second dose of antigen was partly neutralized by reacting with circulating antibodies before it reached the sensitized tissue cells.

**Passive Allergy.**—If the serum of an immunized animal containing, let us say, precipitins is injected into a normal animal, then the tissues of the normal animal adsorb the precipitins after a few hours and are ready to react just as though the normal tissues had produced the antibodies. This is *passive allergy*. In either passive or active allergy, if the conditions are just right, the antibody-antigen reaction in the tissues may result in symptoms and signs ranging in severity from the production of a rash ("hives") or an attack of nausea, to the development of weakness, lowered temperature, dyspnea (difficult breathing), convulsions and sometimes death. Any of these may be symptoms of *allergic reactions*.

The severe and acute type of seizure is called an *anaphylactic* reaction, but all of the other symptoms—simple "hives," nausea, etc.—also result from the allergic state of the patient toward the specific antigen involved.

In the development of allergy, the cells of the body seem to be *oversensitized* by the first injection (*sensitizing dose*) so that the second dose (*toxic dose*) acts like a violent poison or toxin. It seems likely that the symptoms of allergy result, not directly from the antigen-antibody reaction itself, but from some poisonous substance produced in the cells as a result of the reaction. Histamine, or some histamine-like agent may be responsible.

Allergy, like immunity, is highly specific; that is, the toxic dose must be of the same antigen as the sensitizing dose. An injection of strawberry protein will not sensitize to milk or oysters. This specificity is sometimes used in the identification of proteins by tests in animals.

**Desensitization.**—It is a remarkable feature of the more severe and acute type of allergic reaction, especially the form known as the anaphylactic reaction, that once having reacted, the body will not again react in this manner for some days or weeks, for it is not only immune to the antigen which gave rise to the reaction, but temporarily *desensitized* to that antigen *with respect to anaphylaxis*. This, however, is not true of all phenomena of the allergic state. Under some circumstances, persons remain hypersensitive for many years in spite of continuous reactions.

Desensitization, *when practicable* (it often cannot be accomplished), can be achieved by slow stages if several small, successive doses of the antigen be given properly (*e.g.*, 0.1 cc. or lesser doses subcutaneously at hourly intervals). By giving small doses, and by giving them subcutaneously, sudden contact of a massive amount of antigen with the sensitive tissues is avoided and an overwhelming reaction is thus prevented. This is of importance in passive immunity. For example, a patient who, in childhood, received horse-serum diphtheria antitoxin might react very severely in adult life to an injection of horse-serum tetanus antitoxin because, due to his childhood experience, the patient is allergic to horse protein. He should be desensitized before receiving the full dose of tetanus-antitoxin serum. Some sera for tetanus and other treatments are now prepared from goats or rabbits so that if a person receiving them is required to receive horse-serum later, no allergic reaction will occur.

**Types of Allergic Manifestation.**—The reactions of allergy in persons and animals hypersensitive to various antigens are quite varied. Various manifestations of allergy may be described which probably do not differ in principle but only in symptoms, much depending on the kind of antigen, the means by which it gains entrance to the body, the particular tissues sensitized, and the dosage.

**Anaphylaxis.**—This manifestation rarely occurs in human beings but is best seen in guinea pigs. It also occurs typically in dogs and rabbits. In guinea pigs, within one minute after intravenous or intraperitoneal injection of even a small secondary dose of antigen, the animal becomes uneasy and scratches at its nose.

coughs, and is evidently embarrassed for air. Gagging movements occur, and the animal gasps for breath. Urination and defecation take place, the animal falls on its side and ceases to breathe. Death may supervene within a few minutes. If the attack is not fatal, recovery is often abrupt and seemingly complete within an hour or two. Most of the symptoms are believed due to contraction of smooth muscle fibers, large amounts of which are present in the lungs, thus constricting the air passages. The fibers are also present in the intestines and bladder. Reductions in temperature and other signs are probably due to the release of a histamine-like poison by the tissues involved. In rabbits, dogs and other animals the picture varies, probably due to differences in anatomical location of smooth muscle. In pregnant animals abortion often occurs because the uterus consists largely of smooth muscle. In dogs there is much damage to the liver and this alters the clinical picture markedly.

In human beings typical anaphylaxis rarely occurs, but many other manifestations of hypersensitiveness are seen. As a matter of custom, the terms allergy and allergic reaction are applied chiefly to phenomena of hypersensitiveness in human beings, anaphylaxis to reactions in animals. All are closely related but there are certain differences which may justify considering human allergic reactions as a separate group of phenomena. For example, anaphylaxis is an artificially induced condition; allergy spontaneous. Desensitization to anaphylaxis is easy; in allergy it is often difficult or impossible. The nature of the antibodies in allergy (called *reagins*) differs from those in anaphylaxis.

*Pollen Allergy.*—The pollen of many plants, among them ragweed, grasses, roses and golden rod, is capable of producing allergy in some persons. The protein which the pollen cells contain, landing on the oral and respiratory mucosa of these persons, appears to set up a hypersensitive condition of these tissues so that when the person later inhales more of the same pollen, the tissues of the mucous membrane lining his nose and throat become irritated, swollen, and edematous ("spongy" with serum or lymph), and all the symptoms of a severe "cold" may develop, due to the severity of the reaction against the pollen. This condition is very well known and is commonly called "hay fever" or "rose fever." A person may *sometimes* be desensitized by receiving a series of small, hypodermic injections of the proper pollen.

*Food Allergy.*—By an analogous process, a person may become sensitized to a certain food, for example, strawberries or codfish. If for some reason, such as a slight gastro-intestinal irritation, lesion,

or other disturbance, some of the protein of the food gets past the stomach or intestinal wall and into the blood in an *undigested* or partially digested condition, the result is the same as if the protein had been injected or inhaled. The next time, and perhaps every time, the person eats strawberries or codfish, he may react more or less violently. Usually under these circumstances the allergic reaction manifests itself in the form of a rash or blotches on the skin which itch persistently. These are commonly known as "hives." In other cases, the victim may become violently ill and exhibit nausea, vomiting, intestinal irritation, and perhaps other symptoms. Such persons may often be desensitized, as in pollen hypersensitiveness or anaphylaxis, by receiving one or more injections of the protein of the appropriate food.

*Bacterial Allergy.*—Bacteria as well as viruses and other micro-organisms entering the blood stream or tissues may sensitize certain cells of the body to their protein as well as stimulate antibody production. The relation between antibodies and the reactivity of the sensitive tissues is not clear. In many instances the tissues of the skin seem highly sensitive, a condition readily demonstrated by intradermal injection of the antigen, which results in a local redness and swelling. This may be due to the presence of the antibodies in the cells of the skin. A very complicated set of reactions occurs which may cause a variety of symptoms, depending on the kind of tissues, organisms and individual involved.

Whenever an infectious disease becomes *subacute* or chronic, all of the body cells may become sensitized to the bacterial protein, and give rise to various symptoms and little-understood reactions. A person who has been infected with the tubercle bacillus remains in an allergic condition to the organism, an important consideration in regard to his resistance to the disease. Skin tests for allergy to tubercle bacilli are of great value in the study of tuberculosis as will be pointed out later (see page 661). Allergy, therefore, plays an important part in infectious diseases. Other diseases of this chronic nature are syphilis, undulant fever and swine erysipelas.

*Skin Reactions.*—If the quantity of antigen injected as toxic dose is not excessive, there is often manifested a power of the skin to bind and localize the antigen. In such localized reactions necrosis of the skin may follow a marked edema.

*Arthus Phenomenon.*—Among these localized skin reactions is the phenomenon described by Arthus. If a rabbit receives injections of some foreign protein, say monkey serum, over a period of several weeks, the later injections cause ulcers and sterile abscesses



to occur at the site of inoculation. These develop very quickly, and there is no anaphylactic reaction, indicating that the antigen is held in the tissues around the site of the inoculation so that it does not reach other cells of the body.

**Schwartzman Reaction.**—If a rabbit be injected *intradermally* with an antigen, say a sterile, filtered broth culture of certain bacteria, the site of the intradermal injection, although apparently unaffected after the *first* injection, will react violently, with a hemorrhagic and necrotic lesion, when similar material is injected *intravenously* twenty-four hours later. It is as if the tissues at the site of the first injection removed the material later injected into the blood stream, and bound it, with deleterious results locally. The exact mechanism of this reaction is not clear. It is not entirely specific.

**Serum Sickness.**—After the injection of serum for the treatment of disease, such as diphtheria or tetanus, there often occur undesirable reactions due to sensitivity of the body cells to the proteins in the serum. The antitoxin has nothing to do with the reaction, since normal horse serum will cause the same reaction. These reactions may occur in persons not known to have had previous injections of serum, and appear in various forms ranging from acute and serious illness accompanied by collapse and dyspnea coming on a few minutes after the injection, to milder, delayed forms such as arthritis, hives or extensive rashes. The reaction is presumably an allergic one known as "*serum sickness*."

The mechanism is not clear but may be due to the rapid development of precipitins which react with traces of horse serum still present in the patient's blood. As these reactions are most likely to occur in patients who have asthma, investigation is usually made of this point before serum is administered. If the serum is to be given intravenously, a preliminary skin test for hypersensitiveness to horse serum may be made, as described below. If the test is positive, the patient is *desensitized*, as previously described, by giving very small preliminary doses of the serum.

**Skin Tests for Hypersensitiveness.**—The skin of a person or an animal hypersensitive to any given protein will, as shown by Arthus, develop a large, localized, inflamed area where a small quantity of that protein has been scratched or injected *into* the skin. This local reaction is simply another manifestation of an allergic condition, but it is extremely useful in determining what protein is causing a given patient's trouble.

In tests for food or pollen sensitiveness, sterile solutions of vari-

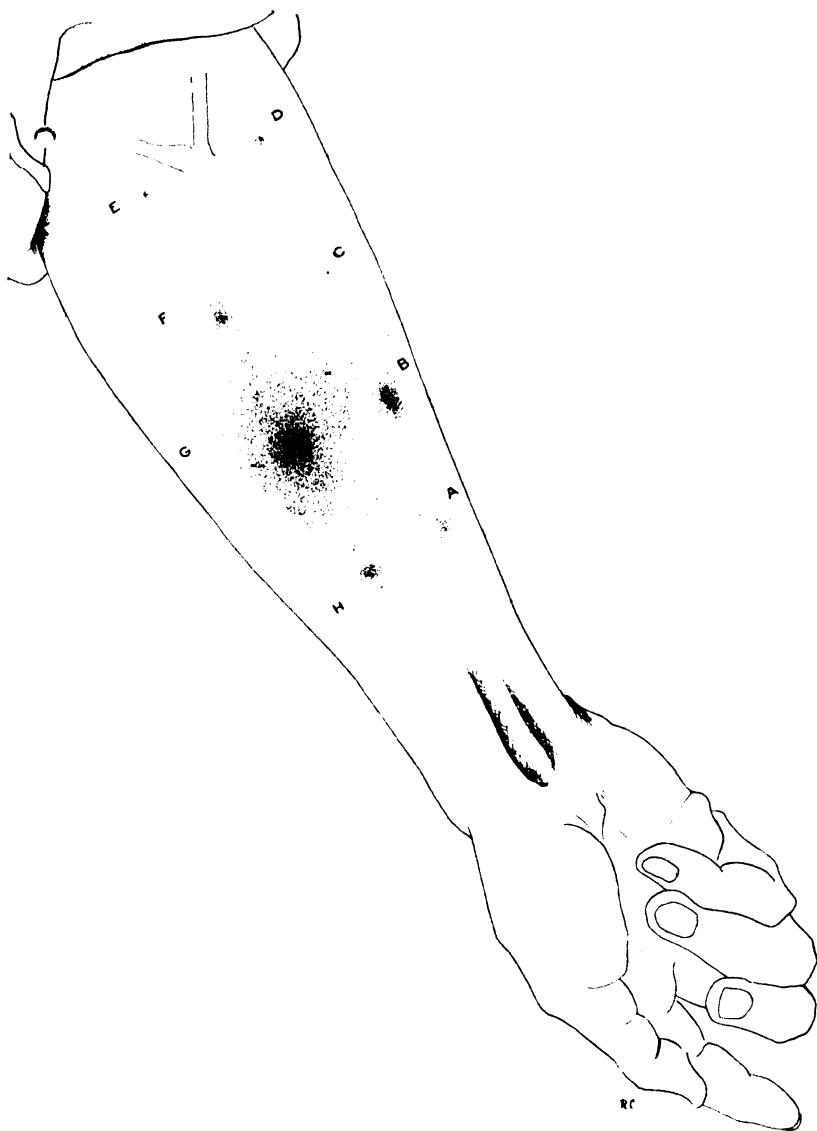


Fig. 158.—Reactions in the skin after intradermal injection of various antigens to test for allergy. *A* milk; *B* pork protein; *C* strawberry protein; *D* hen's egg; *E* codfish; *F* rose pollen; *G* cat dandruff; *H* golden rod pollen. Obviously the patient is hypersensitive to the protein in cat dandruff. The reactions at *A*, *B*, *D*, *F* and *H* are not especially significant.

ous foods or pollens which would be likely to be involved, are introduced *into* (not under) the skin, either by scarification or injection, and the results observed. A number of "likely" substances are usually tested at the same time (Fig. 158). The one causing the most severe reaction is probably the cause of the patient's trouble.

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## CHAPTER 19

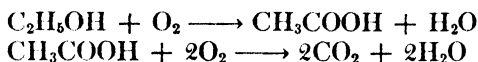
### ENZYMES AS RELATED TO BACTERIOLOGY

THE ORIGINAL sources of our knowledge of cell physiology and of the chemistry of cell metabolism can probably be attributed to the laboratories of early physiologists who were interested in all phenomena related to the human body. Plant cell physiology also had very early beginnings and the studies of plant and animal physiologists supplemented and complemented one another. When biochemists and cell physiologists began to investigate yeasts and bacteria, it was revealed that these lowly creatures had a physiological chemistry as complex as the cells of more highly evolved beings. It was especially interesting to note that the physiological chemistry of animals, human beings and plants of all sorts, including bacteria and other fungi, was fundamentally alike. Different physiological mechanisms were involved, and a variety of chemical devices were made use of in various species and classes and phyla, but the net result of all of these was essentially the same.

Many of the physical and chemical mechanisms underlying plant and animal metabolism are fairly well understood and can be observed and controlled experimentally. Among the most extensively investigated mechanisms are the exothermic reactions, *i.e.*, those involved in respiration or the utilization of foodstuffs as

energy sources. The endothermic reactions resulting in synthesis of protoplasm, *i.e.*, the utilization of foodstuffs as building material for the living organism, are also being subjected to intensive studies but are not yet so well understood as the exothermic reactions. Both the energy-yielding (or respiratory) and the energy-storing (or cell-building) processes are accomplished largely through the agency of organic catalysts\* called *enzymes*. A brief discussion of catalysis and of enzymes may, therefore, be useful in later discussions on bacterial metabolism.

**Catalytic Agents.**—If two chemically reactive substances, as for example oxygen and ethyl alcohol, are adsorbed at ordinary temperatures upon the surface of a third inert substance, such as platinum, which has been treated so as to be in a finely divided or colloidal condition, we have a series of reactions as follows:



facilitated and controlled by surface concentration or adsorption. These reactions take place at an astonishing rate of speed in the presence of the platinum, whereas ordinarily oxygen and alcohol combine exceedingly slowly, even imperceptibly, except at ignition temperatures (burning). The platinum does not enter into the reaction, but remains to adsorb more oxygen and more alcohol, and so continues the process of oxidizing the alcohol first to acetic acid and then to water and carbon dioxide. In such a reaction the platinum acts as a *catalytic* agent, and its action as such apparently depends upon its colloidal state with its large surface area. In a simple system of this kind we can predict, from a knowledge of the substances and surfaces involved, what the result of a given combination will be. Inorganic catalysts are simple and constant and are extensively used in industry. In living systems the situation is more complex. Here several organic catalysts called *enzymes* act simultaneously.

**Enzymes.**—An enzyme is an organic substance of definite chemical and physical structure, probably a protein (certainly always closely associated with a protein) which is elaborated by living cells and which, like inorganic catalytic agents, without permanently combining with the reacting bodies, greatly increases the velocity of certain chemical reactions which would not otherwise occur, or would occur very slowly, at biological temperatures in

\* In order to understand catalytic agents, the student should review the sections on colloids; surfaces of colloids; surface tension; adsorption.

media which are neutral or nearly neutral. About twenty enzymes have been isolated in a pure state. All are proteins. Some are crystalline (Fig. 159).

*Specificity.*—A very distinctive property of enzymes is their *specificity*. This is of almost the same order as immunological specificity and probably depends on similar stereochemical relationships. For example, an enzyme which catalyzes the oxidation of natural *l*-amino acids will not oxidize the *d*-amino acids. Enzymes which hydrolyze proteins will not hydrolyze carbohydrates and vice versa.



Fig. 159.—Crystalline pepsin. (Northrop.)

An enzyme which destroys the carboxyl group of pyruvic and related keto acids will not attack the carboxyl group of acids like acetic acid.

The specificity of enzymes supports the view that their role in catalysis is not the formation of an adsorption compound, as described for platinum, but that the enzyme temporarily combines chemically with the substrate (the substance being acted upon) to form an unstable compound which immediately undergoes the oxidative, hydrolytic, or other change characteristically induced by that enzyme, releasing the enzyme to repeat the process. Thus

a very small amount of an enzyme can bring about a large amount of chemical change in a relatively short time. For example, 2 cc. of concentrated extract from pigs' pancreas, containing the enzyme *trypsin* which hydrolyses protein, can decompose approximately 5 pounds of ground beef within five hours or less at 37° C. and a pH of around 7.5. The ratio of beef to actual, pure enzyme is over a million to 1.

**Classification and Nomenclature of Enzymes.**—Early students of physiology, discovering new enzymes, often gave them descriptive names without reference to any systematic scheme of nomenclature. Such names as *pepsin* (the protein-digesting enzyme of the stomach), *trypsin* (the proteolytic enzyme of the pancreas), *ptyalin* (the starch-digesting enzyme in saliva) are time-honored examples of this early method of naming enzymes. Later it became necessary to systematize nomenclature. It is now customary to name an enzyme by adding the suffix “ase” to the substrate or substance acted upon. For example, protein-digesting enzymes are *proteinases*, *amylase* digests starch (*amylum*), and so on. Some enzymes are named for their activity. For example, *oxidases* bring about oxidations; *dehydrogenases* cause hydrogen to leave the substrate; *hydrolases* cause hydrolysis or digestion. Some enzymes act upon *groups* of substances, as trypsin, a proteinase which acts upon proteins in general. Some proteinases are very specific; for example, bacterial *caseinase* acts only on casein and not on other proteins.

Specificity may consist in difference in *manner of attacking* a given substance. For example, *sucrases* are not all alike. There are glucosido- and fructosido-sucrases, the former attacking the glucose end of sucrose molecules, the latter attacking the fructose end. Glucosido-sucrase occurs in *Aspergillus* species, while fructosido-sucrase occurs in yeast. Such differences may be seen among bacteria, as several species may attack a given substance with different end products.<sup>1, 2, 3</sup>

**Distribution of Enzymes.**—There appears to exist, in one cell or another, plant or animal, an enzyme capable of attacking in some way practically any naturally occurring organic substance. Thus it is difficult to find a natural organic compound incapable of supporting some sort of life under suitable conditions of moisture, temperature, accessory food sources, etc. However, while all living cells contain or produce enzymes which catalyze their vital chemical processes, all cells do not produce all enzymes. Among bacteria there are very sharp and clear-cut differences in this respect, and it is partly by means of their specific and characteristic enzymes

that we identify and classify bacteria. For example, many intestinal bacteria produce *lactase*, but the typhoid bacillus and related species which cause intestinal disease do *not* produce *lactase*, a fact which greatly aids in their isolation and identification.

**Endo- and Exo-enzymes.**—Enzymes may exist only inside the cell, or they may be secreted to the exterior environment where they carry on mainly hydrolytic or digestive functions. Enzymes within the cell are intracellular or *endo-enzymes*, while those functioning outside the cell are extracellular or *exo-enzymes*. Most of the exo-enzymes are hydrolases. Enzymes which are not hydrolytic, mainly endo-enzymes, are often grouped as *desmolases*. This group includes *oxidases*, *dehydrogenases*, *mutases* (cause a Cannizaro reaction), etc. Examples of various kinds of enzymes are seen in the accompanying abbreviated table (Table II) which gives the nomenclature, action, and other information regarding some enzymes.

TABLE II  
ENZYMES (PARTIAL LIST)

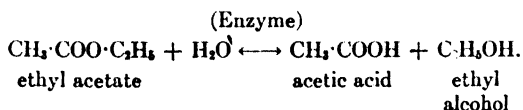
Name	Substrate and Action
<i>Proteinases</i>	<i>Proteins hydrolyzed in varying degrees</i>
Pepsin	Proteins hydrolyzed to proteoses, peptones, etc.
Trypsin	Proteins hydrolyzed to polypeptides and amino acids
Gelatinase	Gelatine hydrolyzed.
Fibrinolysin (bacterial)	Fibrin decomposed.
<i>Carbohydrases</i>	<i>Carbohydrates hydrolyzed</i>
Amylase	Starches hydrolyzed to maltose.
Lactase	Lactose hydrolyzed to glucose and galactose.
Sucrase	Sucrose hydrolyzed to glucose and fructose.
<i>Esterases</i>	<i>Esters, fats, etc., hydrolyzed to alcohols and acids</i>
Lipase	Fats hydrolyzed to glycerol and fatty acids.
Phosphatase	Organic phosphates broken down to phosphate and radicle.
<i>Oxidases</i>	<i>Oxygen activated to combine with substrate</i>
Indophenol oxidase	Oxidize cytochrome and indophenol.
Peroxidase	Release active oxygen from $H_2O_2$ .
Polyphenol oxidase	Oxidize di- and tri-phenols, and related compounds.
<i>Carboxylases</i>	<i>Decompose carboxyl groups</i>
Pyruvic carboxylase	Carboxyl group of pyruvic and related acids split to $CO_2$ and $R\cdot CH$ .
Amino acid carboxylase	Carboxyl group of amino acids split to $CO_2$ and $R\cdot CH$ (bacterial).
<i>Deaminases</i>	<i>Split off amino group of amino acids</i>
l-amino acid oxidase	l-amino acids deaminized to $NH_3$ and aldehyde or keto acids.
Guanase	Guanine oxidatively deaminized to $NH_3$ and xanthine.

This table is incomplete, but serves to show how enzymes are classified and the present method of naming them.

**Reaction Rates and Equilibria.**—It is important to bear in mind at least three fundamental characteristics of enzyme action. *First*, enzymes are believed never to *initiate* reactions that could not occur spontaneously at ordinary temperatures. They appear merely to *facilitate* and *speed up enormously* spontaneous reactions which normally occur *very slowly*, perhaps imperceptibly, at temperatures compatible with life. A classical example is the observation of Berthelot (1862) who noted that, if he placed ethyl acetate in a beaker of water in a warm place, after a *considerable period of time* ethyl alcohol and acetic acid appeared in the solution, their concentrations slowly increasing to a point at which *equilibrium* was reached and the solution finally contained *fixed* amounts of the three substances involved. *Hydrolysis* of the ethyl acetate obviously occurred spontaneously but *very slowly*. If a very little hydrochloric acid were added, the reaction proceeded hundreds of times faster, the hydrochloric acid acting as a catalytic agent and remaining unchanged in the process. A similar observation was made with regard to amyl butyrate; *lipase* from the pancreas, a true enzyme or organic catalyst, enormously facilitated its hydrolysis into butyric acid and amyl alcohol.

*Second*, reactions brought about by many enzymes are *reversible*. Thus, if we add acetic acid and ethyl alcohol to a beaker of water, we observe the combination of a portion of these to form ethyl acetate, the *same point of equilibrium* being reached as in the experiment noted above, but relatively slowly if allowed to proceed spontaneously. The combination occurs much more rapidly if a suitable catalytic agent (HCl) be used. The same is true of amyl alcohol, butyric acid and the enzyme, *lipase*. In many instances reactions of this nature *appear* to proceed in only one direction because the equilibrium point is very far in that direction. In other cases one phase of the reaction seems to occur with greater ease than the reverse.

It is interesting to note that, in hydrolysis, a molecule of water is introduced; in the reverse reaction it is removed:



Other examples of this type of reaction are seen in a later chapter.



The reverse of hydrolysis, it will be observed, results in synthesis, sometimes improperly spoken of as polymerization. Synthetic starch has been made by the action of an enzyme on glucose.<sup>4</sup>

*Third*, enzyme reactions, although reversible and although they embrace both hydrolytic and synthetic phases, under the conditions of action inside the cell are usually pushed decidedly toward the one or the other phase of the reaction. Otherwise matters would tend to become static and this is not compatible with life. Thus, in the hydrolysis of ethyl acetate, equilibrium is soon reached in a beaker of water, but in a living cell the products of hydrolysis may be removed as fast as they are formed and equilibrium may never be reached.

**Composition of Enzymes.**—Although many enzymes are easily demonstrated by their activity free from the living cells which produced them, relatively little is known as to their exact chemical composition. One difficulty in obtaining exact information about many bacterial enzymes arises from our inability to obtain large quantities of them in a pure, native state, and to be certain that what is supposed to be a single enzyme is not a mixture of two or more. However, many enzymes of animals, yeasts and other fungi have been obtained in pure, even crystalline, form. The first so obtained was urease; later crystalline pepsin, trypsin, amylase and about sixteen others were prepared. All that have been purified have been shown to be proteins and to form colloidal solutions. The activity of the enzyme is dependent upon the integrity of the protein molecule.

**Activators. Coenzymes.**—Enzymes sometimes exist in an inactive form called zymogens. These are *activated* by contact with some other agent called a *kinase* or activator. Trypsin, for example, exists in the pancreas as trypsinogen which becomes active when it gets into the intestine in contact with a substance called enterokinase. The esterase phosphatase is activated by magnesium ions. Pepsinogen is activated by metallic ions, bile salts and a variety of other substances to give it full activity.

Many enzymes, for example, yeast zymase, seem to be composed of two parts which are inactive separately; (a) a nondialyzable colloidal protein and (b) a specific, active or prosthetic group or *co-enzyme* which is nonprotein and dialyzable. Some coenzymes are readily separable from the protein molecule constituting the colloidal part of the enzyme, others are very firmly attached and it is doubtful whether they may be considered as separate chemical bodies.

The activity and specificity of the entire complex (called a *holo-enzyme*) seem usually to be conferred in great part by the coenzyme or prosthetic group. For example, the zymase of yeast, a readily dialyzable and separable coenzyme, confers both activity and specificity on the colloidal, nondialyzable protein part of the enzyme, called apozymase. The two together constitute the holoenzyme. On the other hand catalase, another kind of enzyme, consists of a very stable union of a specific colloidal protein with hematin. The colloidal part gives activity and specificity, as shown by the fact that a similar enzyme, peroxidase, is composed also of hematin, but this is combined with a different protein.

**Factors Affecting Enzymes.**—Enzymes, being protein complexes, are chemically and physically unstable and, therefore, sensitive to various influences which affect proteins in general. For example, all enzymes are adversely affected by temperatures above or below their optimum,  $pOH$  or  $pH$ , high osmotic pressures, excessive concentrations of substrate or end products, often by pressures, ultra-violet light, and so on. The activity of many enzymes is destroyed by specific enzyme poisons, among which are narcotics, cyanides, dyes, etc. These poisons are often adsorbed on, or combine with, the catalytically active group of the enzyme to the exclusion of the usual substrate. Some of these "blocking" actions are reversible, others permanently inactivate the enzyme.

Comparing these statements with what has been said of the effect of various environmental factors on bacteria, it is clear that, in general, whatever affects enzymes also affects bacteria (as well as all other living cells), since the physiological chemistry of bacterial and other cells is almost entirely due to, and dependent upon, the action of enzymes or enzyme-like bodies.<sup>5, 6</sup>

**Cell Surface as an Enzyme.**—While the enzymes, both extra-cellular and intracellular, as well as the constituent protoplasm of bacteria are undoubtedly of great importance in metabolism, there is much support for the view advanced by Quastel and his colleagues that many important reactions are brought about by, or on, the external surfaces of the cells themselves. Bacterial cells are so small that they have many of the surface properties of colloidal particles. For this reason it seems not unlikely that the cell itself accomplishes many of the changes ascribed to enzymes by adsorbing and transforming substances on its own surface, the nature of the transformations depending on the chemical and physical make-up of the cell exterior, its electrical potentials and other factors.

This mechanism is not to be thought of as a substitute for the concept of enzymes, but as an additional mechanism for producing metabolic changes.

**Products of Bacterial Metabolism.**—Not all bacteria possess all enzymes. For example, only sulphur bacteria (*Thiobacillus* and *Thiobacteriales*) possess hydrogen sulfide dehydrogenases. Only the genus *Nitrobacter* possesses oxidases for nitrites, and so on. The metabolic compounds resulting from the action of enzymes are, therefore, often characteristic of various species.<sup>7</sup> Indeed, as shown in a previous chapter, it is largely by the study of the products of metabolism that the enzymes are recognized and bacteria identified. The identification of bacteria by their metabolic products or peculiarities is extremely important and is more fully discussed elsewhere (see page 225). Some of the products of metabolism, like ethyl alcohol and acetic acid, are very useful and valuable in industry. Others, like diphtheria toxin, are extremely poisonous. Some of them, like nitrates, are indispensable for plant food and are formed in large quantities only by the action of bacteria which inhabit the soil. Indeed, it is to the bacteria of the soil that the human race owes its existence. If the soil bacteria ceased functioning, plants would not grow, empires and republics, old order and new order, would fall to dust and proud *Homo sapiens* would pass out of existence, leaving the autotrophic bacteria in possession of all territory and quite disdainful of international boundaries or "giant pincers movements." We shall explain how this is in a succeeding chapter.

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## CHAPTER 20

### BACTERIAL METABOLISM

#### CARBON UTILIZATION; HYDROLYSIS; DISSIMILATION

THE LIFE-CHEMISTRY of the Schizomycetes is not different in principle from that of other living things. They require water and a generous supply of certain elements common to all protoplasm, such as C, H, O, N, S, P, Ca, K, and lesser amounts of other elements such as Mg, Cl, Fe, Cu, and Mo. Some of these elements are obtained from complex molecules by means of enzymes which split organic compounds into simpler compounds and elements; some are obtained from  $H_2O$ ,  $CO_2$ ,  $H_2S$ ,  $MgSO_4$ , and so on. Foods may be used in two ways. First, they may be assimilated in the synthesis of cell substance and reserve food like starch, fat, etc. Such uses of food involve *endothermic reactions* since energy, derived as shown below, is "built in" to the synthesized substance and stored there.

Second, all organisms, including bacteria, bring about oxidations (*exothermic or respiratory reactions*) of certain of their food materials which yield the energy manifested as motion, reproduction, heat, synthesis, and so on.

**Autotrophic and Heterotrophic Nutrition.**—On the basis of the kind of material which they can utilize as a source of carbon, all bacteria may be divided into two large but not sharply differentiated groups, autotrophic and heterotrophic.

*Autotrophic bacteria* can thrive on media composed entirely of inorganic substances. They are especially characterized by ability to utilize inorganic carbon ( $CO_2$ ). Many of them also utilize only inorganic nitrogen sources (as  $NaNO_3$ ), and oxidize inorganic compounds like hydrogen sulfide as energy sources.

*Heterotrophic nutrition* is seen in bacteria which are able to use organic compounds, such as glucose, as sources of carbon. They often also require organic compounds, such as amino acids, as sources of nitrogen. Further, they characteristically utilize organic compounds to obtain energy but are not restricted to such compounds.

Numerous species of bacteria are neither strictly heterotrophic nor strictly autotrophic. There are many gradations between strict autotrophism, such as is found in the genus *Nitrobacter*, and the

strictest forms of heterotrophism as illustrated by the fastidious, parasitic gonococci and influenza bacilli.

The different nutritive types of organisms reflect varying abilities to synthesize protoplasm and other complex cell materials from simple nutrient materials. The most highly developed synthetic powers are seen in those autotrophs which require no organic materials yet can build up carbohydrates, fats, proteins, and complicated systems of enzymes including various vitamin molecules, and the like. Other species can get along as well if supplied with an entirely inorganic medium except for a little glucose as a source of carbon, since they cannot build carbon dioxide into complex compounds. Still others, usually parasites, have relatively little synthetic power, and require that their culture media contain, in addition to appropriate minerals, protein like egg or serum, or mixtures of certain amino acids, a number of vitamins which they cannot synthesize, and often glucose as a source of energy or carbon or both.

*Heterotrophic Sources of Energy or Cell Substance.*—One of the difficulties in studies of heterotrophic metabolism is that of knowing which compounds are used in synthetic processes and which serve as sources of energy. With simple autotrophs, or species which will grow in solutions the composition of which is exactly known, it is a relatively easy matter to determine these points by noting the products of oxidative reactions under various conditions or to substitute single sources of nitrogen, or carbon or energy in otherwise complete food mixtures of known composition and look for presence or absence of growth. Simple experiments of this type are described farther on.

When using complex solutions like meat-infusion broth with peptone, one knows little about what serves for cell synthesis and what is used as a source of energy. Consider a culture of *Escherichia coli* in a tube of infusion broth to which lactose has been added. We know that lactose (as well as other materials) is metabolized, and changed into other products. Some of these can be found in the culture after growth but, in the words of Buchanan and Fulmer,<sup>1</sup> “. . . there is no simple means of determining whether the carbon-assimilation [synthesis] needs are satisfied from the original compound [lactose] or from one of the products [such as lactic acid] . . .” Some organisms seem to use the same sorts of substance for both growth and energy but “. . . it is difficult to differentiate sharply . . . what proportion of organic carbon [as in lactose] may be utilized for energy and what assimilated.

"The energy derived [from the metabolism of organic compounds] may be utilized in part in evolution of . . . heat . . . light . . . movement, and in part may be stored as reserve food in the cell." Some light has been thrown on these problems by the use of compounds in which the carbon is radioactive. This is discussed farther on (page 346).

It is significant in this connection that the actual *weights* of bacterial substance obtained with various compounds used as sole sources of carbon are often definitely related to the *caloric* or energy value of these compounds.

**Bacterial Carbon Dioxide Metabolism. Photosynthesis and Chemosynthesis.**—We have spoken of the utilization by autotrophic bacteria of carbon dioxide and water to synthesize carbohydrates, etc., and it is well known that green plants accomplish the same thing. But whereas green plants require sunlight to perform this transformation, autotrophic bacteria (except certain pigmented species) achieve the synthesis in the absence of any light. It has also been shown that many bacteria, formerly thought of as entirely heterotrophic with respect to carbon sources, can also utilize carbon dioxide as a source of carbon for cell growth in the absence of sunlight and that many of them, probably all, while perhaps also requiring complex sources of carbon, absolutely require a certain minimum amount of carbon dioxide in order to grow. Many will grow much better in atmospheres containing from 2 to 10 per cent carbon dioxide.\*<sup>1a</sup> All bacteria, then, may utilize some carbon dioxide for cell synthesis, but most species do so in a manner seemingly different from that of green plants.

Very careful and exhaustive studies by van Niel, Wood, Werkman, Franck, Gaffron<sup>2</sup> and many others indicate that, although heterotrophs do not use carbon dioxide as an exclusive source of carbon, carbon dioxide has an important place in the nutrition of both heterotrophic and autotrophic bacteria, as well as in that of green plants and some (possibly all) animal cells. In all forms of life it now appears likely that hydrogen is removed from water or some other hydrogen-bearing compound like hydrogen sulfide (all of which are spoken of as *hydrogen donors*) and that the hydrogen is used to reduce carbon dioxide to form some organic compound which is the basis of synthesis of organic carbon compounds.

\* A simple but effective method of cultivating bacteria in an atmosphere containing approximately 10 percent CO<sub>2</sub> is to place the cultures in a jar containing a candle, light the candle and close the jar. The candle ceases to burn when the CO<sub>2</sub> content approaches 10 percent.

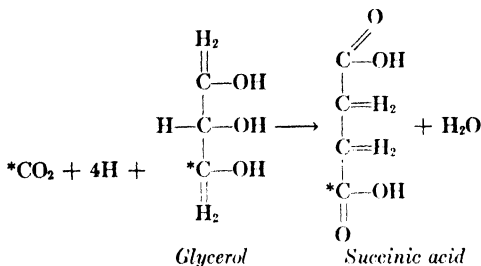
Three important differences are seen between green plants and autotrophic and heterotrophic bacteria in their mode of reducing carbon dioxide. These differences are manifest in (a) *the source of energy used to extract the hydrogen from the donor and transfer it to the carbon dioxide*. In green plants energy is derived from sunlight which is absorbed and utilized in some manner by chlorophyll. In the case of certain pigmented bacteria, sunlight is utilized by a closely related compound called *bacteriochlorophyll*. Cell synthesis under the influence of light is called *photosynthesis*. All bacteria other than the photosynthetic, bacteriochlorophyll-bearing species are devoid of photosynthetic pigment and must obtain their energy for synthesis from respiratory functions involving oxidations. Cell synthesis based on this type of reaction is called *chemosynthesis*.

Chemosynthetic organisms, which include nearly all species of bacteria which are of agricultural, domestic, industrial or medical importance, grow well in the dark and are usually injured by direct sunlight. They differ greatly in respect to the substances which they can utilize as energy sources. Some species (*e.g.*, *Nitrobacter*) can utilize only inorganic substances as energy sources, such as the oxidation of sodium nitrite to sodium nitrate; many others can utilize a variety of organic fuels, such as alcohol or glucose; others, *e.g.*, certain *Cytophaga* species, are restricted to cellulose, and so on.

(b) *The chemical nature of the hydrogen donor*. This may range from molecular hydrogen, through water and hydrogen sulfide, to organic compounds like  $\text{CH}_3\text{COOH}$ , and  $\text{C}_8\text{H}_{16}\text{COOH}$ , depending on the species of bacteria involved. Green plants use water exclusively, liberating oxygen. Many photosynthetic bacteria utilize hydrogen sulfide, liberating sulfur.

(c) *Manner in which the carbon dioxide is assimilated*. In the case of green plants and strictly autotrophic bacteria incapable of using any other source of carbon than carbon dioxide, carbon-to-carbon linkages must be brought about directly between molecules originating from carbon dioxide, as by polymerization of  $\text{CH}_2\text{O}$ . In heterotrophic species not so restricted, there is evidence that carbon dioxide molecules are not always linked directly together but combine with already formed organic molecules. For example, Werkman, Carson, Wood, and their colleagues have "fed" carbon dioxide made with "heavy" carbon ( $\text{C}^{13}$ ) and radioactive carbon ( $\text{C}^{11}$ ) to propionic acid bacteria. The special carbon can be followed, like a tracer bullet, through its course in the synthetic processes of the bacterial cell. For each molecule of the radioactive carbon

dioxide, one molecule of succinic acid or propionic acid is formed by the organism from a molecule of glycerol.

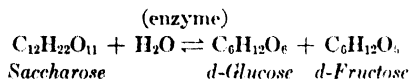


The use of such "tagged" atoms has provided one of the most useful tools for elucidation of the general problem of the utilization of carbon.<sup>3</sup> This in turn may one day enable us to understand how green plants perform, under our very noses, one of the great mysteries of life—the utilization of carbon dioxide and water to form such material as sugar, starch, wood, etc.

### BACTERIAL DIGESTIVE PROCESSES

Bacteria, it has been pointed out, are strictly holophytic and cannot take through their cell membranes any solid matter, or even any very large molecules. Yet they are capable of utilizing as food dead leaves, the trunks of fallen trees and other plant matter, as well as crab shells, hair, horn, flesh of dead animals and other organic debris. The agents by means of which they accomplish these wonders are enzymes, notably *carbohydrases*, *esterases* and *proteinases*, which are *hydrolytic* in their action.

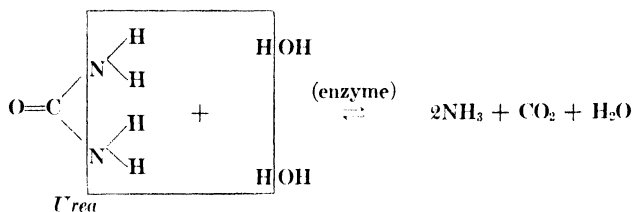
These bring about the decomposition of large, complex molecules such as those composing chitin of crab shells, cellulose of wood, gelatin, coagulated blood, flesh, fat, etc., into smaller ones, soluble in water and capable of diffusing through the cell membrane so as to be acted upon as food by the intracellular catalysts. In this way, by extracorporeal digestion as it were, bacteria can utilize large masses of solid matter as food. Since these hydrolytic decompositions occur outside the cell, any energy released is lost as far as its usefulness to the cell is concerned. Examples of hydrolytic reactions are the splitting of cane sugar into *d*-glucose and *d*-fructose:



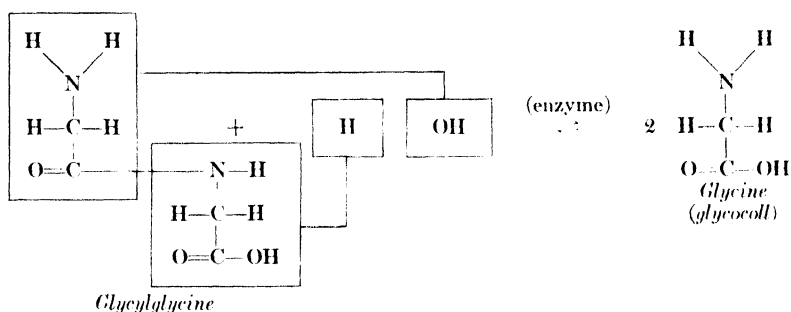
\* Radioactive C.



or the breaking-up of urea into ammonia and carbon dioxide:



or the division of 1 molecule of the polypeptid glycylglycine into two of glycine:



Note that a molecule of water is introduced into the molecule of substrate in all hydrolytic reactions.

**Carbohydrate Hydrolysis. Cellulose Decomposition.**—Excellent examples of the various types of reactions cited in the foregoing are found in the scavenging of various organic wastes in the soil, for example, in the decomposition and metabolism of cellulose and other plant carbohydrates.

Consider the trunks of enormous trees that fall; consider the tons of leaves that accumulate each autumn; consider the mountains of vegetable wastes from canneries, and the tons of cornstalks, straw, stubble and vegetable matter which are plowed into the soil. What happens to all of these? We say, "They decay or putrefy." But what is this? The material composing the rigid, woody parts of plants as well as the skeletal structure of soft parts is principally cellulose, or a dense material of the same nature called "ligno-cellulose," combined in various ways with gums, starch, waxes, pectin, and the like. The ligno-cellulose and cellulose are complex carbohydrate materials chemically related to starch; *i.e.*, they are polysaccharides. Wood, of course, is relatively resistant to water, weather, cold, weak acids, alkalis, and other influences and will

withstand them for considerable periods, sometimes years. However, soil microorganisms decompose wood rapidly.

Certain species of bacteria and a great many kinds of molds and larger fungi produce enzymes which *hydrolyze* ligno-cellulose and cellulose into molecules of simpler carbohydrates like hemi-cellulose and also into cellobiose. Cellobiose, in turn, is hydrolyzed by a considerable number of bacteria and molds into simple molecules of glucose. Still other organisms (or perhaps the same ones) attack the starches, etc., in various plant structures and hydrolyze them, producing, in the case of starch, successively erythrodextrin, achroodextrin, dextrins, maltose and finally glucose.\*

In the decomposition of cellulose in the soil, each successive and simpler derivative of the cellulose serves as a source of carbon or energy, or both, for organisms which continue the decomposition of the derivative available to them in their own characteristic manner.

The final products of the decomposition depend on the sorts of bacteria and molds present in the particular soil under consideration and on whether abundant oxygen is present or not. If air is present in abundance, highly oxidized substances such as carbon dioxide and water are generally the chief end-products, with "humus" remaining as a soft, brown substance so desirable in fertile soils and slowly undergoing the last stages of the decay process. In case abundant air is not present (as in the muck of boggy or swampy places or heavy clay soils), more "intermolecular" and "intramolecular respiration" or *fermentation* (see next chapter) is likely to occur, with the development of reduced and partly oxidized end-products such as methane, hydrogen, alcohols, and butyric and other organic acids which contribute to the acidity of "sour" soils.†

The hydrolytic decomposition, in the soil, of a complex substance such as wood somewhat resembles the physical dismantling of a large mansion. First, doors, window-sashes, stair-rails, fireplace mantels, and so on, are removed. Then whole sections of roof, wall or flooring may be taken down. (All these parts would correspond to rather large molecules like cellobiose and various dextrins into which the cellulose, starch and other polysaccharides are at first split.) It may be that neighboring builders can utilize these same

\* A similar process can be brought about by boiling wood, corn cobs and similar substances with acid for long periods. (See section on industrial alcohol production, page 587.)

† The draining and liming of such soils is necessary for agriculture since most of the bacteria (especially the genera *Nitrobacter*, *Nitrosomonas* and *Nitrosococcus*, and the genera *Azotobacter* and *Rhizobium*, all of which will be discussed) are sensitive to acid and are aerobic.

doors, window frames, stairs and roof sections intact. This is an efficient use of material and energy. This would correspond to the utilization by various bacteria of the split compounds—dextrins, cellobiose and others—resulting from the progressive splitting up of the woody material. Other smaller and simpler constructors may require that the larger sections of the house be broken down to their constituent boards and bricks before making use of them. This would correspond to the final hydrolysis to glucose, of which nearly all the other carbohydrate molecules are built, and its utilization by being further split and metabolized by still other bacteria.

**Dissimilation. Glucose Metabolism.**—A good example of the end stage of cellulose metabolism is seen in the decomposition or dissimilation of the glucose into which it is largely hydrolyzed by various common soil bacteria.<sup>4, 5, 6, 7, 8</sup> As we have seen, glucose (or other carbohydrate) may serve both as an energy source and as a source of carbon for cell synthesis. It is difficult to determine just how the organisms arrange these matters. However, we can survey by chemical means the “left-overs” after the organism has metabolized the carbohydrate, and we may add chemical indicators or traps of various sorts to the growing cultures to detect substances which, although not appearing among the end products, may be formed temporarily during cell metabolism. Several of the intermediate products in glucose dissimilation have been isolated or identified by precipitation or colorimetric methods or by determining the effect of adding certain supposed intermediate substances, for example acetaldehyde, on the amount of derivatives of acetaldehyde produced. Important and very common among these intermediate products are *pyruvic acid* and *acetaldehyde*. In addition, we know (or *think* we know) all of the end products resulting from glucose metabolism by certain species so that, with our knowledge of some of the intermediate, and all the end, points we may plot out such a sequence of chemical steps as that shown below for an hypothetical species (Fig. 160).

This schema is simplified to exclude certain series of reactions in which the glucose first combines with phosphoric acid, the triose appearing as a triose-phosphoric acid compound. Some other intermediate steps have also been omitted. The diagram is not as complicated as it looks. It shows that glucose splits into two triose molecules, a transfer of hydrogen taking place from one part of the glucose molecule to the other. This is called *intramolecular respiration*. It is also spoken of as molecular rearrangement. One molecule

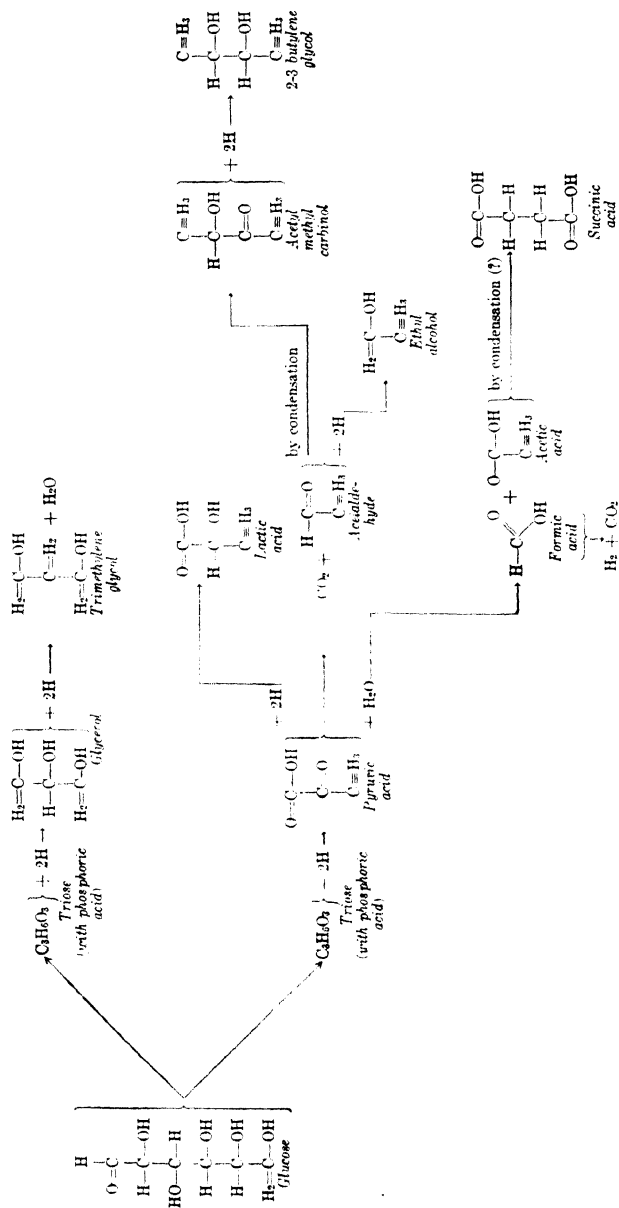


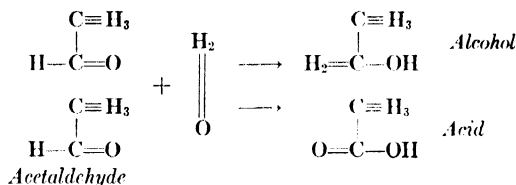
Fig. 160.—Final stages in decomposition of cellulose after the cellulose has been hydrolyzed to glucose molecules.

of triose is then used as a hydrogen acceptor (hydrogen is derived from elsewhere in the metabolic processes of the cell) and is reduced to glycerol which in turn accepts more hydrogen and becomes trimethylene glycol.

Another molecule of triose may be oxidized by dehydrogenation, forming pyruvic acid, a sort of chemical turntable capable of undergoing a variety of changes some of which are shown. As a hydrogen acceptor it may be converted to lactic acid. It breaks down directly to form carbon dioxide and acetaldehyde. Part of the latter may and probably does undergo aldol condensation to form acetyl-methyl-methyl carbinol which is in turn reduced to 2,3-butylene glycol. Another part of the acetaldehyde may be reduced to ethyl alcohol. The combination of pyruvic acid with water to form formic and acetic acids is somewhat doubtful. Probably the two acids are derived from the pyruvic, but it may be that the hydrogen and oxygen are not derived from water as such.

Some doubt also exists as to how the succinic acid is formed. It may be derived by condensation of two molecules of acetic acid as indicated or it may be derived by the transformation of pyruvic with carbon dioxide and dehydrogenation.

It may also be that dismutation occurs between 2 molecules of acetaldehyde to form alcohol and acetic acid, thus:



This is a Cannizzaro reaction, an example of *dismutation*. Mutases (produce dismutation) are well known enzymes capable of producing this type of interaction between water and a variety of substances and are found in many types of cell.

In the case of *Aerobacter aerogenes* the following final metabolic products of glucose have been demonstrated to occur: hydrogen, carbon dioxide, ethyl alcohol, acetic acid, acetyl-methyl-carbinol, 2,3-butylene glycol, trimethylene glycol, lactic acid, glycerol and succinic acid. *Acetaldehyde*, *pyruvic acid* and *acetyl-methyl-carbinol* have been demonstrated as intermediate, transitory substances. Whether the reactions that occur in their formation are all exactly as indicated is not certain, but many seem probable. One curious point is that there is seldom *exactly* the same quantitative relation-

ship between the end-products. Sometimes there is much carbon dioxide and little hydrogen, sometimes the reverse. Observe also that one sees molecular rearrangements, oxidations, reductions and condensations all going on at once.

One group of reactions not indicated in the schema includes the combination of glucose with phosphoric acid (phosphorylation) prior to its splitting up. Werkman and others,<sup>9</sup> have shown that phosphoric acid forms intermediary compounds in the process, such substances as *phosphoglyceric acid* having been isolated during glucose fermentation by some bacteria. Phosphopyruvic acid is probably also formed. The role of phosphates in the metabolism of sugars by yeasts has been very thoroughly studied and there is a tendency to assume that what is true of yeast is also true of bacteria. It is in part true for some species but may not be true for all.

As shown above, some of the end-products of glucose metabolism are acids and alcohols, which may eventually kill the bacteria in the culture. Some of the acids may under certain conditions of aeration be broken down further so that after a time the reaction of the culture reverts to alkalinity partly through the formation of carbonates. This is common in the *Salmonella schottmülleri* group.

Some of the products of bacterial metabolism as diagrammed are valuable in industry, ethyl alcohol and butyric acid for instance. *Aerobacter* has its own distinctive end-products by which it may be distinguished from closely related species. One of these is acetyl-methyl-carbinol which is easily tested for in a culture tube. It also has commercial value. Other organisms may often be identified by analogous tests.

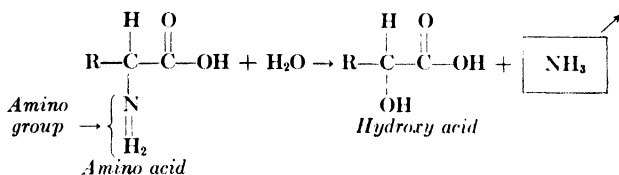
**Protein Hydrolysis.**—Protein is built up of amino acid molecules much as complex carbohydrates are made up of glucose molecules. They are linked in the manner shown for the combination of glycine units in glycylglycine. Amino acids are the "building stones" of protein or protoplasm. Hydrolytic enzymes (proteinases) first split proteins into large "sections" which are, chemically, albumoses, proteoses, and polypeptides of various degrees of complexity (glycylglycine is a simple polypeptide, a dipeptide). This hydrolysis is analogous to the splitting of wood into hemicellulose, cellobiose, dextrans and so on. The larger nitrogenous molecules (albumoses, etc.) are further and further decomposed by hydrolysis until finally the amino acid stage is reached. Hair, horn, hoof, insect and crustacean shells (chitin), flesh, all of which are protein or compounds related to protein, are similarly decomposed, primarily by hydrolysis, into simpler substances available as food.

**"Protein-sparing" Action of Glucose.**—Proteolytic enzymes, in general, are produced most abundantly and act best in fluids of alkaline reaction. If much glucose or other "fermentable" substance be present, the acids formed from its metabolism greatly interfere with proteolytic processes. It was formerly thought that the bacteria "preferred" the carbohydrate and used it in preference to the protein, the glucose thus seeming to exert what is often referred to as a "protein-sparing" action. It has been shown that if the acids are neutralized by buffers or alkali as fast as they are formed, protein is attacked vigorously in the presence of glucose.

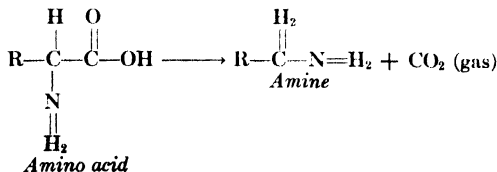
This hydrolysis, similar to that of complex carbohydrates, further resembles it in that the end-products of complete hydrolysis are relatively simple units—glucose in the case of carbohydrates, amino acids and related compounds in the case of proteins and related substances. In either case the cell deals most directly with the glucose or amino acid. We have seen how certain kinds of bacteria deal with glucose. Let us see what sort of reactions occur in the final utilization of amino acids. It must be re-emphasized that amino acids, like glucose, may serve as sources of both energy and nitrogen, and probably carbon, oxygen, sulfur, etc., as well, and that our knowledge of how the cell does all these things is still fragmentary.<sup>10, 10a</sup>

**Amino Acid Dissimilation.**—Various schemata have been drawn, analogous to that for glucose decomposition by *Aerobacter*, suggesting how the amino acids may be broken down and utilized. Here again, the end-products give some clue to the nature of the processes. Various intermediate products of the reactions involved have been isolated or demonstrated, and others plausibly postulated. Four hypothetical types of reaction may be listed as follows:

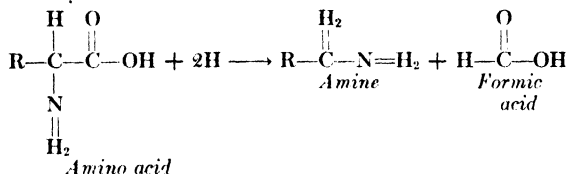
- (a) Hydrolysis, resulting in deamination with liberation of ammonia:



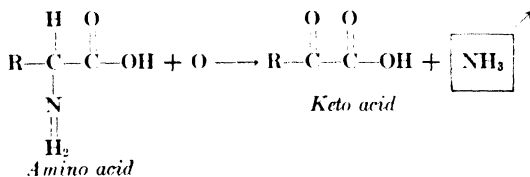
- (b) Decarboxylation (a molecular rearrangement):



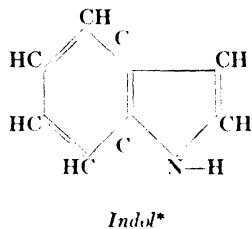
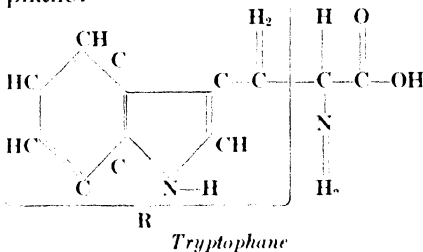
(c) Hydrogenation (reduction):



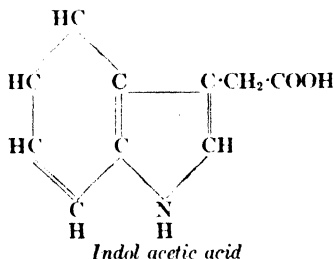
(d) Oxidation (with deamination or liberation of ammonia):



In these formulae R in the amino acid may be no more than a  $\text{CH}_3$  group, or it may be a very complex radicle like that in tryptophane:



The oxidative deamination of tryptophane results in the formation of indol acetic acid which is a potent growth hormone for many green plants, favoring root growth especially.

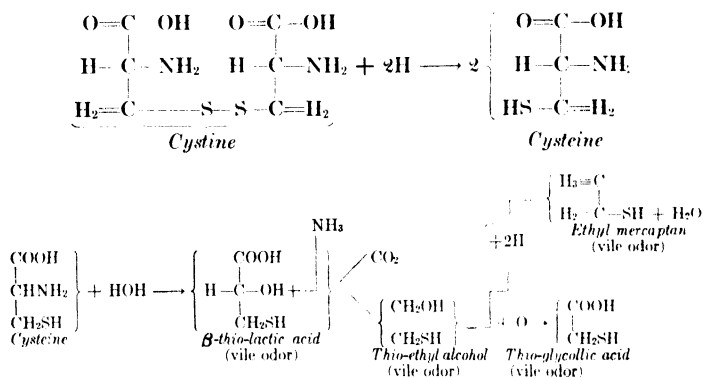


\* The molecule of indol is placed beside that of tryptophane for comparative purposes. Some important bacteria are identified by the fact that they can split off the indol group from the tryptophane molecule.



Note that, in two of the reactions given above, (a) and (d), ammonia is split off. This is a very common occurrence in protein decomposition, and helps to contribute to the odoriferousness of the process and also to the fertility of the soil containing it (see sections on *Nitrosomonas* and nitrogen cycle, pages 406 and 413). The keto and hydroxy acids also contribute to the odors of protein decomposition. Other foul odors commonly sensed in putrefaction of protein are due to volatile sulfur compounds—the mercaptans, skatol, hydrogen sulfide and others.

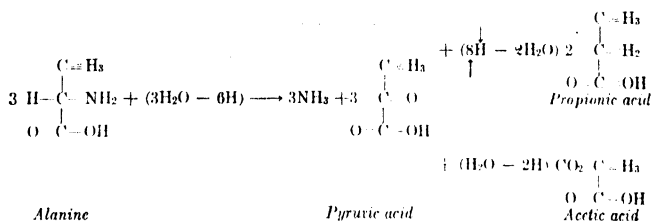
These may be derived from sulfur-containing amino acids, like cystine, as follows:



The reactions in this schema are largely hypothetical but the end products have been demonstrated in part.

Some of the amines (reaction *b*) produced as a result of protein decomposition were formerly believed to be poisonous if eaten and were called *ptomaines*. This is very doubtful.

A *hypothetical* schema showing one way in which an amino acid such as alanine *may* be decomposed and used as a source of carbon and energy is of interest because the products are much the same as those resulting from glucose metabolism by some bacteria.



Hydroxy acids like lactic acid, and similar compounds, undergo similar changes.

Not all bacteria decompose the same amino acids, nor do they attack amino acids in the same manner. As in carbohydrate decomposition, tests for certain end-products like indol (see above) are used in the identification of certain species of bacteria.

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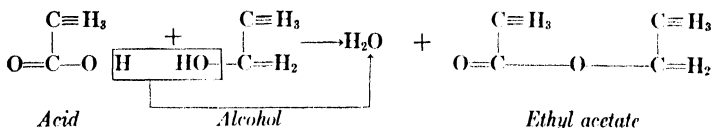
## CHAPTER 21

### BACTERIAL METABOLISM (Continued)

#### ASSIMILATION; RESPIRATION

**Synthetic and Assimilative Reactions.**—In cell synthesis from organic materials certain of the atoms and molecules into which the food substance is first divided by hydrolytic and other enzymes as described in the last chapter, are rearranged, combined with various minerals, etc., and put together again in new structures constituting the protoplasm, cellulose, fat, glycogen, chitin, volutin, and other portions of the organism. The reactions involved are synthetic and are *endothermic* since energy is absorbed and stored in the complex molecules resulting from the synthesis or building process. Many of the endothermic reactions are the reverse of hydrolysis, sometimes called polymerization or condensation, although these usages are not exactly correct chemically. When the endothermic reaction results in the synthesis of actual cell substance, *assimilation* is said to have occurred.

As pointed out in the section on enzyme action, some hydrolytic changes are reversible, and complex molecules are in part built up from simpler ones by the abstraction of a molecule of water. It is not unlikely that in some instances the same enzymes bring about the changes in either direction, the conditions in the cell determining the direction of the reaction. A simple type of synthetic reaction is seen in the formation of ethyl acetate from acetic acid and alcohol:



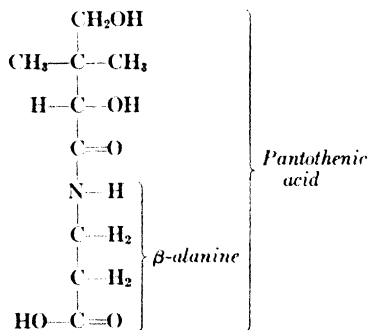
or in the building of polypeptides from amino acids (see page 353).

**Growth Factors.**—Growth factors are substances which do not yield energy to the cell nor contribute materially to its bulk, but which are absolutely essential to its continued well-being. Vitamins are good examples of growth factors, as well as certain metallic elements. Certain specific amino acids have the status of *growth factors*; that is, they are among the substances absolutely essential to the growth of a number of bacteria. Methods of determining these facts have been discussed. A good example is trypto-

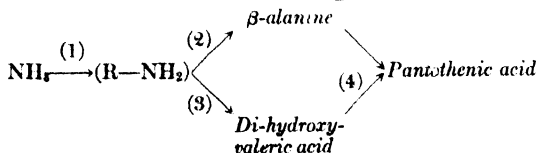
phane, without which no growth of the typhoid bacillus (*Eberthella typhosa*), the lockjaw organism (*Clostridium tetani*), the diphtheria bacillus (*Corynebacterium diphtheriae*) and several others can occur, even though their culture medium be complete in all other respects. On the other hand, there are many bacteria which can synthesize their own tryptophane and so do not need to have it fed to them. Among these are autotrophic bacteria, *Pseudomonas aeruginosa* (a soil and water saprophyte) and numerous others which, though grown in a tryptophane-free medium, nevertheless synthesize this amino acid.

It is probable that such critical amino acids play a role in the building of protoplasm analogous to that of vitamins, *i.e.*, they are a part of a vital component in the cell. A number of other critical substances or growth factors are known or are under investigation. Among them are nicotinic acid, riboflavin, thiamin, pantothenic acid and other vitamins.<sup>1, 2</sup>

A probable role of such compounds or growth factors in bacterial nutrition was described by Fildes.<sup>3</sup> Assume that an organism requiring pantothenic acid, a vitamin, is unable to synthesize  $\beta$ -alanine (one of the amino-acid radicles in, or associated with, pantothenic acid),



but is able to carry out all other syntheses, beginning with simple ammonia, necessary to the formation of pantothenic acid (see 1, 3, and 4 in the diagram below). Given a minimum quantity of  $\beta$ -alanine, completion of the pantothenic acid is provided for and growth occurs. Without  $\beta$ -alanine, no growth is possible.<sup>9</sup>



A strain or species able to synthesize  $\beta$ -alanine as well as the other components of pantothenic acid encounters no such difficulty and we say that "it does not require  $\beta$ -alanine." Both may require  $\beta$ -alanine, but one can manufacture it internally, the other cannot.

**Exothermic Reactions. Respiration.**—Portions of the food substances or their constituent molecules not used in synthesis may undergo oxidations or respiration which liberate the energy required for the syntheses described above, as well as for vital processes such as motion, reproduction, spore formation, and so on.

**Aerobic and Anaerobic Metabolism.**—Since oxidations serve as a source of energy, it might be supposed that all bacteria require free oxygen. Many species, like *Pseudomonas fluorescens* and *Bacillus subtilis*, are of this type. They cannot get along without free access to air. These are spoken of as *strict aerobes*. However, other species which utilize atmospheric oxygen can also grow quite well without free oxygen. These are called *facultative anaerobes*. They use combined oxygen for cell synthesis. Many common species, like *Escherichia coli*, *Streptococcus lactis* and *Staphylococcus aureus*, are facultative in respect to oxygen. Still other species, such as *Clostridium tetani*, *Actinomyces pseudonecrophorus*, and *Bacteroides* sp., not only are able to grow without free oxygen, but absolutely require its entire absence and are very adversely affected by its presence. Such species are called *strict* or *obligate anaerobes*. Pasteur was first to discover this phenomenon. He called it anaerobiosis ("life without air.") Special apparatus, some forms of which are discussed farther on, is necessary for pure culture studies of strict anaerobes in the laboratory. It may be noted that heterotrophic, autotrophic and intermediate metabolic groups may contain aerobes, anaerobes, or species which are facultative.

Some bacteria seem to be adapted to an oxygen tension somewhat less than that of the atmosphere, yet greater than that of complete or almost complete absence of oxygen. Such organisms are said to be *microaerophilic*.

**Anaerobes, Hydrogen Peroxide and Catalase.**—The sensitivity of strict anaerobes to free oxygen has long puzzled bacteriologists but may be explained on various bases, although the exact and complete truth of the matter is not yet available and probably differs for different species. One explanation is as follows: many bacteria give off hydrogen peroxide as a metabolic by-product when cultivated in the presence of free oxygen. As is well known, hydrogen peroxide is unfavorable to many bacteria and is often used as a disinfectant. Most bacteria, then, would soon succumb to their own deleterious by-product had they not some mechanism for protecting themselves from it. Many bacteria able to grow in the presence of air actually do protect themselves from hydrogen peroxide by the production of the

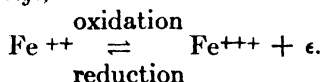
enzyme *catalase* which decomposes hydrogen peroxide as fast as it is formed. The strict anaerobes, on the contrary, produce no catalase, hence they are killed by their own metabolic product when cultivation is attempted in situations where there is more than a certain, minimum amount of free oxygen. There are some aerobic bacteria which form hydrogen peroxide yet which do not produce catalase. But it is found that these species are relatively insensitive to hydrogen peroxide.

Some of the strictest anaerobes may be cultivated when freely exposed to air provided the medium contains some substance which possesses a *strongly reducing*, or oxygen-combining, power which takes care of the excess oxygen so that peroxides are not formed in bactericidal amounts.

*Sources of Energy for Bacterial Growth.*—Molecular or atmospheric oxygen is the final oxidizing agent for all aerobic organisms. Anaerobic growth, however, necessitates that energy be obtained without the use of free oxygen. Nevertheless, oxidations or respiration must furnish the energy of both aerobes and anaerobes. Here we run into a question of terms and definitions. We must redefine the term biological oxidation to include all chemical reactions which liberate energy *available to the cell*.

**Respiration and Biological Oxidation.**—The older physiological terminology was based largely on animal physiology and the term *respiration* is frequently used interchangeably with oxidation because in animals atmospheric oxygen is inhaled by means of the respiratory apparatus (breathing) and is thought of as combining with food-stuffs which are thus “burned” or oxidized in the body. Later, as a result of Pasteur’s studies on fermentation in the absence of air, it became evident that such respiratory functions were not the only means by which living cells obtained energy, yet the term *respiration* continued to be used to signify any exothermic reactions having an energy value for living organisms, whether free oxygen and a respiratory apparatus like lungs were involved or not. In speaking of cellular respiration it has become customary to use the term biological oxidation, and its chemical connotation in the newer sense is quite broad and includes both aerobic and anaerobic respiration.

Fundamentally, oxidation means the loss of electrons, as by change of valence, *e.g.*,



Reduction, conversely, implies the gain of electrons. For example, silver in solution (*i.e.*, in the form of  $\text{Ag}^+$  ions) is not in an obviously oxidized form. Yet, to demonstrate its oxidized condition, it is necessary only to *reduce* the ion  $\text{Ag}^+$  with formaldehyde or glucose,

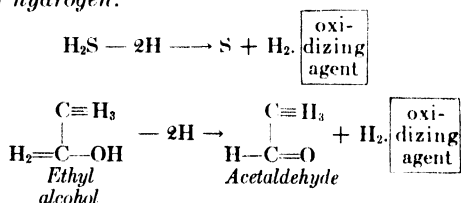
*e.g.*, add electrons to it, to change it to un-ionized, metallic Ag and have it precipitate on a glass surface as is done industrially to make mirrors. It is important to note that in bringing about the reduction of the silver some other substance, which gives up its electrons, becomes oxidized. This compensatory give and take is also characteristic of biological respiratory reactions. An oxidation of one thing implies a reduction of something else, and vice versa and, as oxidation is always going on during life, living creatures maintain a definite *oxidation-reduction potential* in the cell and in their immediate environment, the reducing intensity of which depends on the species, its age, variation phase, and the environment. Since these reactions always involve electron transfers we are able to study them by means of instruments capable of measuring electrical potentials (potentiometers).

In this connection we may consider four kinds of reaction which may be regarded as typifying biologically significant oxidations.

1. *The loss of an electron (change of valence).*

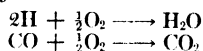
This is in accord with the facts outlined above (see page 361). The oxidizing agent is here serving as an electron acceptor. Any transfer of electrons accomplishes both oxidation and reduction. These changes always occur together. The electron is finally attached to some appropriate acceptor which becomes reduced. It cannot remain free.

2. *The loss of hydrogen.*

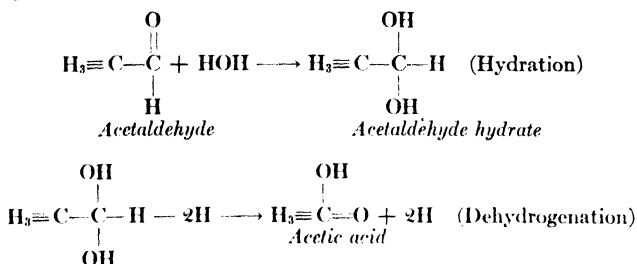


The hydrogen combines with some acceptor (oxidizing agent) such as atmospheric oxygen or other reducible substance. As in example 1, an electron is transferred (at the time hydrogen is transferred) so that an energy transfer is involved.

3. *The addition of oxygen.*



In this sort of reaction either free atmospheric oxygen (free oxygen being used only by aerobes and facultative species) or some oxide or readily reducible compound like sodium nitrate may serve as the oxidizing substance.

4. *Hydration and dehydrogenation.*

Reactions of type 4 are really a combination of the types of reaction given in 2 and 3.

## MECHANISMS UNDERLYING BIOLOGICAL OXIDATIONS

The physiological systems which carry the various types of exothermic reaction to completion are complex and include the respiratory enzymes and several coadjutant bodies. The enzymes themselves, as previously stated, generally consist of a colloidal protein, which may or may not have the property of specificity, combined more or less firmly with a less complex, nonprotein, noncolloidal body called a prosthetic group which generally, but not always, has the property of specificity. Here we shall discuss enzymes as complete structures.

**Respiratory Enzymes.**—The most important respiratory types of enzyme are *dehydrogenases*, *oxidases*, and *peroxidases*. Several other agents will be mentioned which cannot be definitely classified as enzymes but which have accessory functions. All are involved in oxidation or reduction processes, or both.<sup>4, 5, 6, 7, 8</sup>

**1. Dehydrogenases.**—These cause hydrogen to leave the substrate. All dehydrogenase systems involve a *hydrogen donor* (the substrate being oxidized) and a *hydrogen acceptor* (a substance being correspondingly reduced) in order to complete the exothermic transfer of hydrogen. There are two kinds of dehydrogenase systems, which differ significantly in respect to the acceptor of hydrogen.

*Aerobic dehydrogenases* activate\* the hydrogen of the substrate and cause it to combine either directly with molecular oxygen,

\* The exact meaning of this word is not entirely clear but we may think of it as indicating an induced instability of hydrogen so that it readily leaves the molecule of which it is a part and combines with some other substance; or as an instability of any substance so that it becomes chemically active. The explanation is a major problem in biochemistry.



forming water or hydrogen peroxide, depending on the species of cell involved, or with some other readily reducible compound such as sulfur, forming hydrogen sulfide, or with sodium nitrate, forming sodium nitrite and water.

*Anaerobic dehydrogenase* systems do not make use of molecular oxygen, but they transfer hydrogen to an intermediary "respiratory middleman" called a *hydrogen carrier*. This accepts hydrogen from the substrate (in the presence of the dehydrogenase) and transports it toward a final acceptor, such as carbon, sulfur, or nitrogen, often through the intermediation of a second carrier or several successive carriers. Each carrier has the property of accepting hydrogen from a more reduced compound and of being itself in turn dehydrogenated by a component in a less reduced state. These linked systems of hydrogen transfer have been referred to as "physiological bucket brigades." Anaerobic dehydrogenases never produce  $H_2O_2$ , but may produce  $H_2S$ ,  $NH_3$ ,  $CH_4$ , etc.

*Diaphorase*.—This important and widely distributed enzyme, apparently a true dehydrogenase, seems to catalyze the removal of hydrogen from reduced dehydrogenases called pyridine-nucleotide enzymes (page 365). It is present in yeast, *E. coli*, *B. subtilis* and *Proteus vulgaris* as well as in animal tissues. Its chemical nature seems to be that of a flavoprotein, that is, a firm combination of a protein with a yellow-colored prosthetic group (see page 365). It transfers hydrogen from reduced pyridine-nucleotide enzymes to a carrier like glutathione.

**2. Oxidases.**—These may either activate molecular oxygen as hydrogen acceptor, the activated oxygen being reduced to hydrogen, peroxide, or they may catalyze a direct combination of molecular oxygen with some substrate. Two outstanding properties of oxidases are that (a) their activity is inhibited by low concentrations of the poisons potassium cyanide and hydrogen sulfide; and (b) they can act only in the presence of *free* oxygen, that is, they are obligately aerobic.

Examples of oxidases are (a) cytochrome oxidase (indophenol oxidase) which is very common and is found with cytochrome enzyme in virtually all aerobic cells; and (b) ascorbic acid (vitamin C) oxidase. The latter plays an important role in the physiological activity of vitamin C.

**3. Peroxidases.**—These either decompose the hydrogen peroxide formed by oxidases (see above), liberating free oxygen to the air (catalase), or decompose the hydrogen peroxide but, unlike catalase, activate the oxygen so that it combines with readily

oxidizable substrates. Outstanding properties of peroxidases are (a) their oxidative function is inhibited by potassium cyanide; and (b) they often act in *very low* concentrations of hydrogen peroxide so that lethal hydrogen peroxide accumulation due to the activity of oxidases does not occur. (See section on anaerobiosis, page 511.)

**Coenzymes.**—As pointed out previously, enzymes appear to consist of two components, a protein and another group which may be loosely or firmly bound to the protein body, one or both taking part in oxidation and reduction changes. Where separation has been possible, the nonprotein components have been found to have certain physical properties in common. In general they differ from complete enzymes in that they are heat-stable, water-soluble, and dialyzable (*i.e.*, they are smaller molecules than enzymes). They are complex organic compounds and are active participants in enzyme reactions but they are not complete enzymes. Some can be synthesized. They are closely related to, or contain, vitamins. These nonprotein portions of enzymes are called *coenzymes*. They are also known as the *prosthetic group* of their respective enzymes.

Respiratory enzymes may be conveniently classified according to the chemical nature of their prosthetic groups. Here we shall discuss five different prosthetic groups, each part of a specific respiratory enzyme. These prosthetic groups or coenzymes are (1) the pyridine nucleotides, (2) thiamin pyrophosphate, (3) alloxazine-adenine-dinucleotide, (4) the iron porphyrins and cytochrome, and (5) the flavins.

1. *The Pyridine Nucleotides.*—(*Coenzymes I and II*, or diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively.)—These coenzymes are readily separable from the protein component of their enzyme complex by washing or dialysis. They accept hydrogen from substrates whose hydrogen is activated by dehydrogenases, later giving it up to some other substance. Specificity appears vested in the coenzyme and not in the protein moiety. Their composition (Fig. 161) is known from the fact that on hydrolysis they yield:

1. 1 mol nicotinic acid amide (hydrogen-combining radicle),
2. 1 mol adenine,
3. 2 mols pentose,
4. 2 or 3 mols phosphoric acid.

They are readily reoxidized (dehydrogenated) by oxygen or other substances which take their hydrogen from them and which thus

carry forward the respiratory hydrogen transportation initiated by the enzyme-coenzyme combine.

It is of interest to note that some bacteria, *e.g.*, *Staphylococcus aureus*, *Lactobacillus arabinosus*, various strains of *Salmonella*, etc., are able to synthesize part of coenzyme I but must have the nicotinic acid amide, which is a part of it, given them in a preformed condition in order to complete the process. Nicotinic acid, nicotinamide, or other isomers, may or may not serve, depending on the synthetic powers of the species; *H. influenzae* seems to require the entire coenzyme molecule in a preformed state and cannot use nicotinic acid to manufacture it.

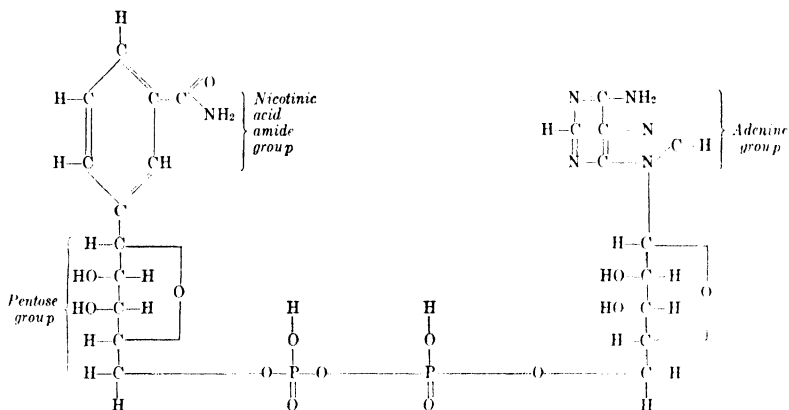


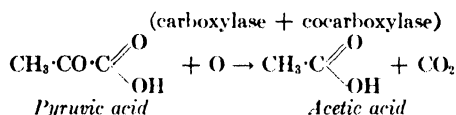
Fig. 161.—Coenzyme I. This is attached to a protein body.

In human beings affected with pellagra (a disease due to nicotinic acid deficiency), administration of nicotinic acid alone is sufficient to produce a marked increase in the amount of *coenzyme* in the blood and to allay the symptoms of the disease, so that the role of nicotinic acid in human metabolism seems fairly indicated as a chemically active radicle or part of the coenzyme molecule in cell respiration. Dysentery bacilli apparently do not metabolize glucose normally in the absence of nicotinic acid, and seem to be injured by the sugar in its absence. Indeed, the vitamin appears to play a decisive role in carbohydrate metabolism by most cells and the reason appears to be its critical importance as a part of the respiratory coenzymes I and II, since carbohydrates serve as sources of energy through the action of respiratory enzyme systems.<sup>10-18</sup>

2. *Thiamin Pyrophosphate (Coccarboxylase).*—This coenzyme is associated with the enzyme carboxylase of various cells (yeast, bac-

teria, etc.) in the decomposition of the carboxyl group  $\begin{array}{c} \text{O} \\ \parallel \\ \text{C} \\ \diagdown \\ \text{OH} \end{array}$

especially of pyruvic acid. Its exact function in the process is not clear. One form of the decarboxylation reaction is oxidative and therefore respiratory in character.



Cocarboxylase is widely distributed in animals, yeast, vegetables, bacteria, etc. It has been artificially synthesized and consists of thiamin linked with two phosphate groups (Fig. 162). It shows

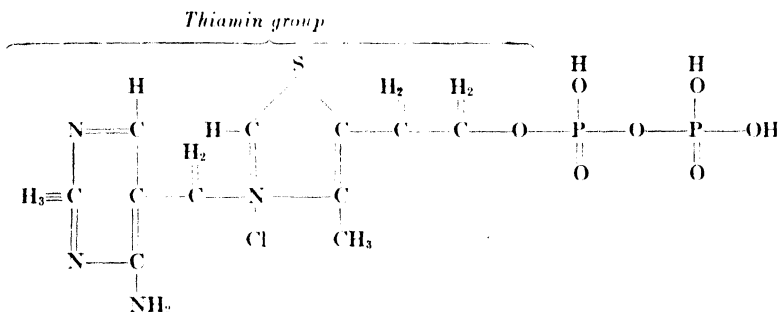


Fig. 162.—Cocarboxylase.

vitamin B<sub>1</sub> (thiamin) activity and maintains normal carbohydrate metabolism in animals. This is because decarboxylation of pyruvic acid is an essential step in the metabolism of glucose by many types of cell. For example, if pyruvic acid accumulates in the blood of human beings we have the disease known as beriberi. In thiamin deficiency of animals, the pyruvates accumulate in the blood, causing similar serious disturbances.

In studies of bacteria, pyruvic acid and acetaldehyde have been shown to occur during glucose fermentation and the function of cocarboxylase may tentatively be inferred as essential in the decarboxylation of the acid, producing the aldehyde. Some species, *e.g.*, *E. coli* and acid-fast bacteria, can apparently synthesize cocarboxylase from inorganic substances with glucose; others, *e.g.*, species of *Propionibacter*, require thiamin preformed in their medium.<sup>2, 15, 19, 20</sup>



not oxidized directly by atmospheric oxygen but transfer hydrogen from some system such as diaphorase to a *carrier* which is finally oxidized by the oxygen, forming hydrogen peroxide.

4. *Iron Porphyrin*.—There are several enzymes of the cytochrome group, a, b and c, of which the most important seems to be c. The coenzyme or prosthetic group of cytochrome is a yellow pigment containing iron in a porphyrin nucleus. This nucleus is attached to a protein body (Fig. 164). The porphyrin coenzyme may be split from the protein by treatment with acid. Cytochrome c is one of the most widely distributed and most important respiratory enzymes. The porphyrin iron, which is the active

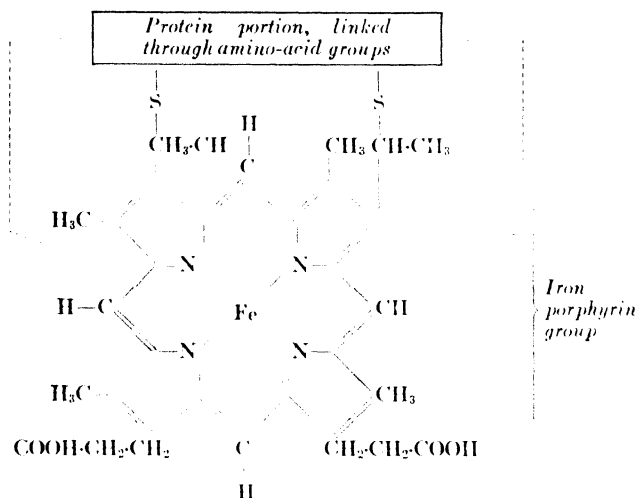


Fig. 164—Probable structure of porphyrin coenzyme of cytochrome C.

oxidation-reduction element, changes valence during oxidation and reduction of the enzyme. Cytochrome accepts hydrogen from a substrate and the final oxidation of the hydrogen by molecular oxygen is then catalyzed by the equally widely distributed enzyme, cytochrome oxidase, but hydrogen peroxide is not produced.

Cytochrome is involved in one half to two thirds of the aerobic respiration of tissues as well as of many bacteria. The porphyrin of cytochrome and hematin belong to a large class of hematin-like pigments, some containing other metals. Among these pigments is chlorophyll, which contains magnesium. They are widely distributed in nature and have functions in respiration.

5. *Flavins (Yellow Enzymes)*.—The flavoproteins have the properties of true enzymes. The yellow flavin or prosthetic group, which may be riboflavin (vitamin B<sub>2</sub>) or a similar substance, is attached to a colloidal protein which differs according to the source of the enzyme. Diaphorase and deaminase may be considered as flavoproteins. Yellow enzymes, *i.e.*, flavoproteins, occur in greatest concentration in cells capable of anaerobic growth. Riboflavin, a well known example of flavins, is present in considerable amounts in eggs and milk. It is the prosthetic group of lactic flavoprotein and has been synthesized (Fig. 165).

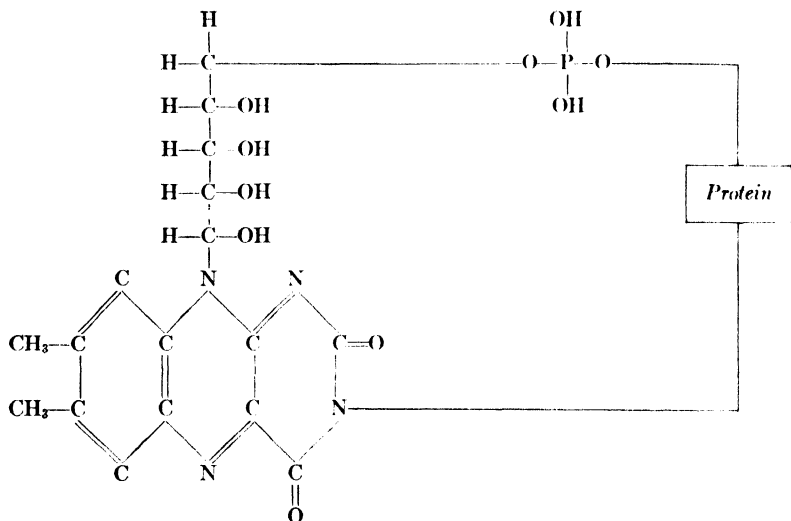


Fig. 165.—Riboflavin. (Compare with Figure 163.)

Riboflavin, or a related flavoprotein complex, is absolutely essential for the growth of some bacteria and may be quantitatively assayed by their growth and metabolism. For example, *Lactobacillus casei* E produces acid from glucose in proportion to the amount of riboflavin present in the medium (see section on *Lactobacillus*, page 599). Flavoproteins function only in dehydrogenase systems, and appear usually to accept hydrogen from coenzymes I and II and to pass it to another substance, possibly cytochrome, or molecular oxygen in some instances.

**Carriers.**—These are complex organic substances, but are, in general, simpler and more heat-stable than enzymes. They are not component parts of enzymes as are coenzymes. They act as hy-

drogen carriers by virtue of being readily reduced by the acceptance of hydrogen, and oxidized by yielding hydrogen.

1. *Glutathione*.—This is a heat-stable compound, the biologically significant part of which is the sulfhydryl ( $-\text{SH}$ ) grouping (Fig. 166), since this gives up or acquires hydrogen readily, thus playing a respiratory role.

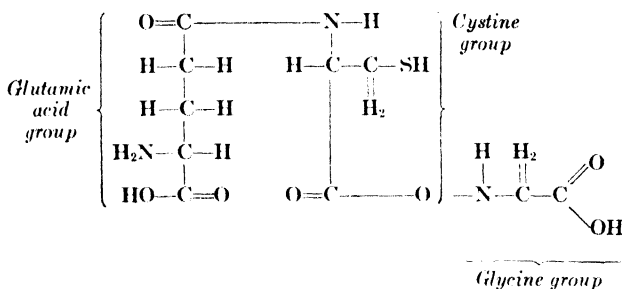


Fig. 166.—Glutathione.

Glutathione is widely distributed in animal tissues and in bacteria. Its exact role in bacterial respiration has not been clearly demonstrated but it may act as a carrier, for example in the formation of lactic acid from pyruvic acid (see schema on page 351). Its action is presumably much like that of cystine since it contains the cystine

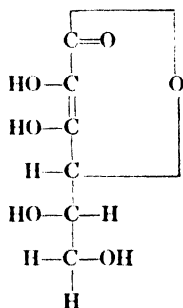


Fig. 167.—Ascorbic acid.

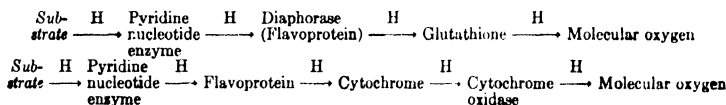
radicle with its  $-\text{SH}$  grouping. In the presence of iron compounds like cytochrome it is rapidly oxidized (dehydrogenated) by molecular oxygen. Thus, a hydrogen donor ( $\text{R}\cdot\text{H}$ ) gives its hydrogen to glutathione (which we may represent by the symbol  $\text{GS}$ ), leaving  $\text{R}$  oxidized and producing  $\text{GSH}$ . If pyruvic acid acts as hydrogen acceptor from glutathione, the pyruvic acid is changed into lactic



acid and GSH becomes GS again. Glutathione probably also plays some role in preventing excessive and destructive oxidation of ascorbic acid (when this is present) and probably other related compounds, since it has a definite reducing action as it exists in the cell.

2. *Ascorbic Acid (Vitamin C)*.—Vitamin C is chemically relatively simple (Fig. 167). Its exact role in bacteriology is not clear. Its antiscorbutic properties and probably other functions, such as protection of the blood complement, are doubtless related to its chemical reducing property. It is destroyed by excessive oxidation. Ascorbic acid may act as a coenzyme for the oxidation of glutathione, *i.e.*, it may accept hydrogen from glutathione.

Respiratory systems involving enzymes and accessory compounds like those described above vary in complexity and in the nature of the compounds involved. Each organism is probably different from all others in the exact details of its respiratory arrangements so that we cannot generalize. In bacteria the whole picture is still very incomplete. However, for the sake of visualization, a diagram of a hypothetical respiratory system may not be amiss. Several of the compounds shown may or may not occur, depending on species of organism, but have been included to show their probable relationships to others. We may consider the following:



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Thaxter, one of the first to study the slime bacteria, described them in 1892 as “. . . a bright, orange-colored growth occurring upon decaying wood, fungi and similar substances, which, although in gross appearance it seemed somewhat highly organized, was found, when examined in a presumably mature condition, to consist of apparently amorphous material without signs of hyphae or spores of any kind.”<sup>1</sup>

**Life History of Myxobacterales.**—The life history of the slime bacteria is divided into two stages or periods. The first (“swarm

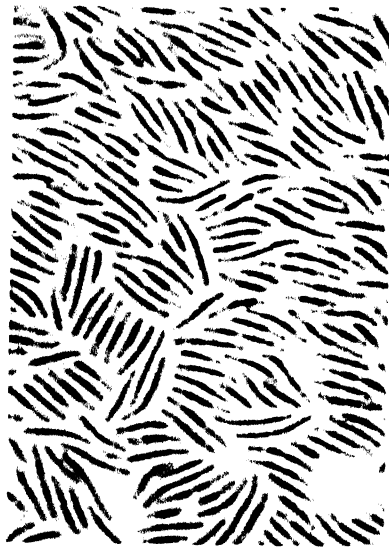


Fig. 168.—A species of Myxobacterales (*Myxococcus xanthus*). Vegetative cells, showing vegetative reproduction. Gentian violet-iodine;  $\times 1860$ . (Beebe, J. Jour. of Bact., Vol. 42.)

stage”) is a period of active multiplication of the rod-shaped, bacterial cells; the second is a period in which they become quiescent (“encystment stage”) and enclosed in cysts of dried slime in which they are disseminated by wind and water.

**The Swarm Stage.**—Starting with a single cell or small groups of, say, two or three cells, we find them multiplying actively by fission and secreting a slimy matrix in which they all live together in the so-called “swarm stage.” Each cell is reddish in color, but the exact nature of the pigment is unknown. The cells are tapered rods, reaching a length of from 10 to 15 microns and a diameter of 0.5 micron

The presence of a definite cell wall is doubtful. They contain granular masses of undetermined character (Figs. 168 and 169, A). Some

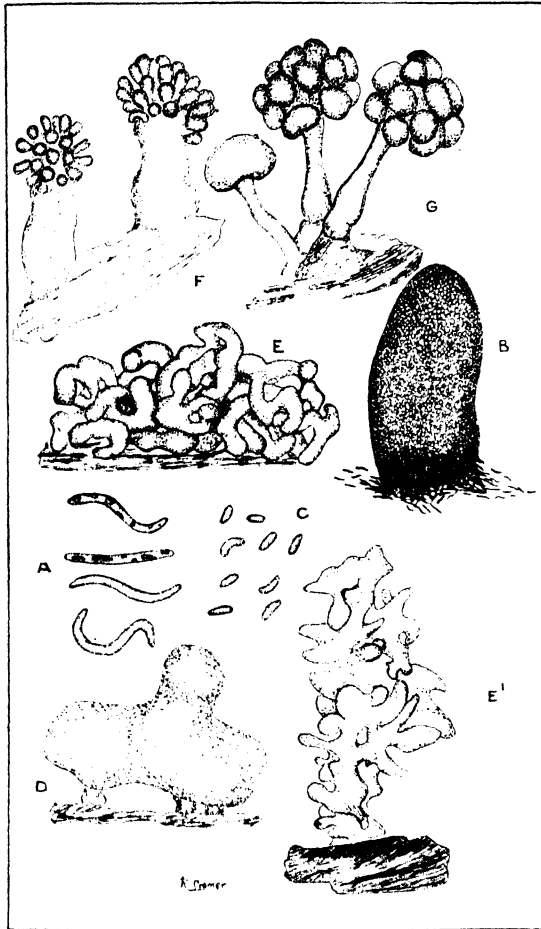


Fig. 169.—Various forms of Myxobacteriales. A Vegetative rods; B one form of spore mass rising from rod mass at its base; C short and rounded rods from a mature cyst; D low, saclike form of fruiting body (*Archangiaceae*); E and E' mature cysts of *Archangium serpens* and one other type; F and G cystophores and budding cysts (probably *Polyangiaceae*). (Redrawn from Thaxter.)

observers believe a primitive nucleus to be present but this is doubted by others. Oil droplets and volutin granules have been

observed. The rods differ from ordinary bacilli in being highly flexible, often bending themselves into loops and in this respect resembling the Spirochaetales. The cells move forward also, by means of a slow, gliding motion. In this stage resemblance to some of the swarming species of *Cytophaga* is striking, so much so that some authorities would include the cytophagas with the slime bacteria.<sup>2, 3</sup> No cilia are present.

All the cells in a colony move forward together, secreting slime as they go, and possibly deriving their motion from this secretory process, more slime being secreted posteriorly than anteriorly, a mechanism suggestive of the Caulobacteriales or stalked bacteria. "A distinct, firm, hyaline, gelatinous base is secreted by the colony as it extends itself, over which the individuals may move or in which they may become imbedded, and is so coherent a structure that whole colonies may be stripped intact by means of it, from the surface of nutrient agar, for example." "In all cases the individuals of a colony are heaped together in the region of its advancing margin which is distinctly elevated above its surroundings, and characteristically roughened by great numbers of partly free individuals projecting from its surface" (Thaxter). This vegetative swarm stage lasts for periods varying from a day or so to a week.

*The Encystment Stage.*—Then begins the period of fructification or encystment. The rods begin to gather together at different points in the margin of the slimy matrix and to heap themselves up. In some forms (Fig. 169, *B*) encystment proceeds directly from this heaping up of cells. The heaps become raised above the substratum, often as papillate projections (Fig. 169, *F* and *G*), the rods pushing to the uppermost portion. They become shortened and rounded, (Fig. 169, *C*) and although they may be referred to as spores or sporoids, they are not very heat resistant. Similar bodies formed by some species of *Cytophaga* are called microcysts. Sometimes large numbers of rounded cells occur together in a cyst. The gelatinous slime dries as an envelope about them, enclosing them in a "fruiting body" within which they resist drying and other unfavorable conditions. These fruiting bodies may be as large as 0.1 mm. (100  $\mu$ ) in diameter.

Various degrees of complexity in the heaping-up and cyst-forming process are observed. In one of the most complex and beautiful species (*Chondromyces crocatus*), the masses raised above the substratum continue to pile up, the base of the rising mass becoming constricted and modified into a slender, tubular stalk, often spirally twisted, as much as 1 mm. in height, and of a bright yellow color.

Within this stalk of slime the cells proceed upward. The stalk becomes branched, often quite elaborately, and on the tips of these branches the cysts are formed by further subdivisions as ovoid "fruits" (Fig. 170). The rods continue to migrate into the cysts,

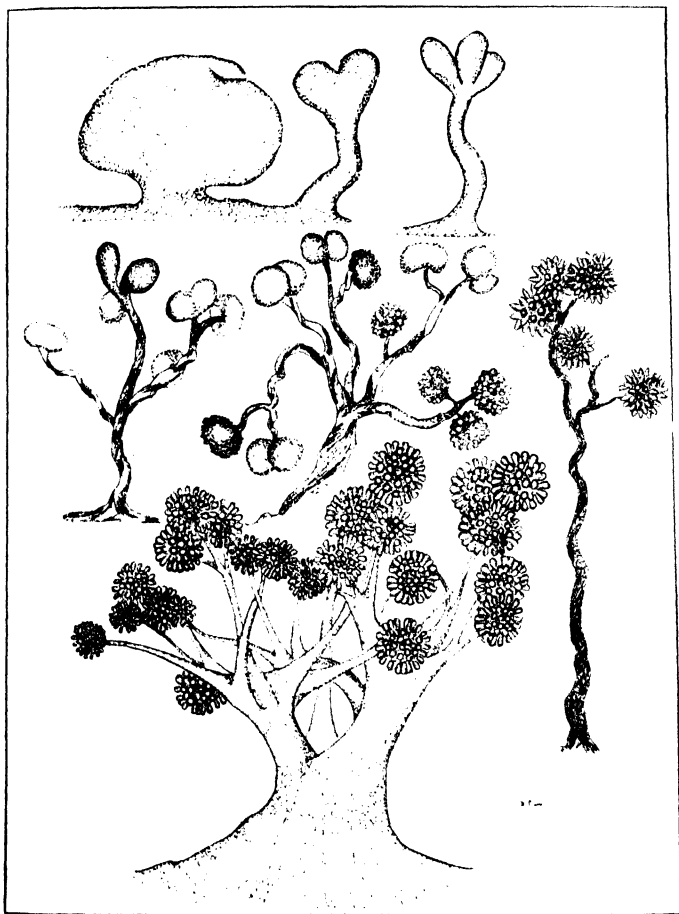


Fig. 170.—Various mature forms of Myxobacteriales. (Redrawn from Thaxter.)

which then dry and are abstricted from the tip of the *cystophore* and disseminated by wind and rain. The cysts are often brightly colored when growing—red, yellow or orange—and, in large groups, give a very striking appearance to the organisms. After a resting

stage, the cysts soften and the vegetative rods emerge as new swarm stages, leaving behind the empty shell of the cysts.

**Classification of the Myxobacteriales.**—When the rods enter the cyst stage, they tend to become much shortened and rounded (Fig. 169, *C*). In one group, the cells become so short and round that they resemble cocci or the spores of bacteria. This group comprises the family of Myxococcaceae. Three other families are listed, namely, the Archangiaceae, in which the fruiting bodies are formed as low, irregular, coiled masses and finger-like projections or columnar bodies, but are *not stalked* (Fig. 169, *B*, *D* and *E*); the Sorangiaceae, in which *angular* cysts are formed but in which *stalks* are *absent* (Fig. 169, *E'*) and the Polyangiaceae in which the cysts are rounded and may be enclosed in fruiting bodies or borne separately on the tips of *branched stalks* (Fig. 170).<sup>4</sup>

**Pathogenic Species.**—At least one species of Myxobacteriales, *M. columnaris*, causing a disease of fish and of considerable economic importance, has been described.<sup>5</sup> It seems likely that other species may be found as parasites on plants and animals.

**Cultivation of the Myxobacteriales.**—The cultivation of these organisms is not difficult. One method is to place pellets of sterilized rabbit feces close together on the surface of sifted, fresh soil in covered dishes and to keep the whole quite moist for a week or two at about 35° C. Many species grow well at lower temperatures; from 10° to 20° C. Pure cultures of some species may be obtained on rabbit-dung agar or on infusion agar. Species found growing on rotting vegetation or dung are best cultivated on material resembling that on which they are found. The exact physiological requirements of the organisms are not well known. Peptone seems to be important, but dextrose of little use. Species vary in their acidity requirements; a fairly wide range is found in some. The field is an interesting one for the student of cryptogamic botany.

**Relationship to Other Forms of Life.**—From a practical standpoint, most of the Myxobacteriales are of little importance. From a systematic standpoint, however, there is much of interest to be found in the order Myxobacteriales. We have already indicated certain points of similarity between the Myxobacteriales on the one hand and the Spirochaetales and certain protozoa on the other, as illustrated in the flexibility and active bending motions of the rods found in the swarm stage of the myxobacteria. The translatory motion of the individual rods of slime bacteria is suggestive of the same type of motion found in sulfur bacteria (*Beggiatoaceae*), in diatoms (*Navicula*), and algae (*Oscillatoria*), while the secretion of slime.

a striking character of the Myxobacterales, is also found in the Cyanophyceae, in true bacteria, in the iron bacteria and in the sulfur bacteria. The remarkable communal existence of the Myxobacterales, in which many cells aggregate for the common aim of transportation, reproduction, and dissemination, is reminiscent of higher plant or animal life, as exemplified in *Volvox* and *Pandorina*, although there is no differentiation of functions among the cells of slime bacteria.

In this connection it is worthwhile to mention a very interesting example of communal motility as seen in certain species of true bacteria of the spore-forming, aerobic genus *Bacillus*. An example is *B. alvei*, described by Shinn.<sup>6</sup> Another of these organisms, *B. rotans*, described by Roberts, is of particular interest in view of the superficial resemblance of the motility of its colonies to that of colonies of the slime bacteria.

"The outstanding characteristic of the organism is the mobility of young colonies on the surface of nutrient agar entirely free from excess moisture. This mobility expresses itself in two characteristic forms. In certain colonies a clearly visible rotary motion occurs, whereas in other cases the entire colony, consisting of thousands of cells, may exhibit a migratory mobility, moving over the agar at the rate of 0.01 mm. per second." "... motility expressed itself first through unorganized, slowly milling masses of cells which finally synchronized their motion and produced either levo- or dextrorotary plates." "In the majority of cases the periphery was broken by the moving cells before a round colony was formed. The release of the cells from the inner portion allowed the colony to move over the agar with a rapid migratory mobility (Fig. 171). Such colonies assumed the shape of a bullet with a round but somewhat pointed



Fig. 171.—*Bacillus rotans*; colony on agar. (J. L. Roberts, Jour. of Bact., Vol. 29, 1935.)



anterior portion and a deeply concave posterior portion" (Roberts).<sup>7</sup> One cannot help wondering if this motile colony might not represent a primitive attempt at communal motility which, however, finds greater expression in the higher order of Myxobacteriales.

In the development of a common, slimy colony and tall fruiting stalks with a resistant encysted stage, we find a surprising degree of similarity to those beautiful creatures, the *Mycetozoa*\* ("slime animals"). Let us briefly consider the latter in order to make the closeness and, incidentally, the superficiality of the resemblance clearer.

**The Mycetozoa.**—A swarm stage is found, which consists of a mass of *living protoplasm* (not inert slime) in which are many *nuclei*. These masses are capable of ameboid motion and, like true amebae, can ingest *solid particles* of food. These are distinctly *animal* characters. The creatures live on rotten logs, etc., and move about in the moisture and shade like amebae. After several days of such existence they cease to move and reproduction begins. The protoplasm sends up *stalks* on the tips of which *sporangia* (spore-bearing cysts) are formed, in a great variety of the most graceful and delicate forms and of the most brilliant colors. Each sporangium contains many spores and each spore contains a single nucleus and is surrounded by a *cellulose* wall. The spores are dispersed by the wind. In water, each spore germinates, forming a naked, ameba-like creature, which later develops a single flagellum. These flagellate forms multiply rapidly by cell division. They then lose their flagella and later *coalesce* to form the *multinucleate swarm stage*.

Thus, in the formation of a common motile colony, and fruiting stalks, we find strong resemblances between slime bacteria and slime animals. But, whereas the individuals of the *Mycetozoa* pass through a stage definitely animal-like, being motile, flagellate and ameboid, the rods of the myxobacteria always remain definitely bacterial rods. Further, the coalescence of a number of ameba-like definitely *nucleated* slime animals into a *single, living plasmodium* has no parallel in the slime bacteria. The "pseudoplasmodium" of the latter is merely lifeless slime, separate from the cells but in which individual cells live. The entire mass of the plasmodium of the *Mycetozoa* is *living protoplasm*. It is a multinuclear, ameboid cell. An illustrated account of the *Mycetozoa* was published in "Popular Science" magazine.<sup>8</sup>

\* *Mycetozoa* are sometimes incorrectly called *Myxomycetes*. *Myxomycetes* is another name for slime bacteria or Myxobacteriales.

The resemblance between these two forms of life is so close, yet so superficial, as to suggest the idea that a comparative study in morphology and function might have been in progress. It is as though Nature had attempted the evolution, in the plant kingdom, of creatures able to live in colonies and disseminate themselves and multiply by encystment; and had tried the same scheme in the animal kingdom along almost exactly parallel lines, the result being a surprising likeness of form in each kingdom.

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#### CHAPTER 23

### THE "SHEATH-FORMING BACTERIA" AND "STALKED BACTERIA"

THE SO-CALLED "sheath-forming bacteria" are, in many respects, among the most interesting organisms which we have to study. They are grouped together in the order Chlamydobacteriales because of the presence of a definite mucilaginous sheath in which the bacteria enclose themselves (*chlamydo* = sheath-forming).

The Chlamydobacteriales are unique among the Schizomycetes in the possession, by most species, of an extraordinary capacity for abstracting iron from ferruginous waters and depositing it in their outer sheath, where it quickly oxidizes to ferric hydroxide ( $\text{Fe}(\text{OH})_3$ ); hence their common name, "iron bacteria." However, it may be

that not all of the Chlamydobacteriales are true iron bacteria for *Sphaerotilis* is said not to deposit iron in its sheath.

Although the iron bacteria are the most outstanding among the organisms which accumulate certain elements or compounds on their exterior, they are not by any means the only ones that do this. The diatoms, which surround themselves with silica so that cell growth is eventually hampered, are suggestive of the iron bacteria which encumber themselves with iron or manganese. Among the algae, the *Zygnemaceae*, *Conferva*, *Cladophluhora*, *Oedogonium* and *Desmids* have been described as accumulating aluminum, iron or chromium, or all three, while *Anthophysa vegetans*, a protozoan, is also active in collecting iron. Certain molds have also been shown to collect iron. The utilization of iron or any other element, in the synthesis of protoplasm must, of course, be carefully distinguished from its mere deposition in the exterior membranes.

Among the most interesting considerations related to these bacteria are their remarkable physiological properties, their place in the evolutionary scale, their role as geological agents and their part in interfering with man's attempts to provide himself with pure water. None are pathogenic; but several, as *Sphaerotilis* and *Crenothrix*, cause difficulties in water supplies (see pp. 385, 388.)

Bergey's Manual lists four genera in this family, namely, *Leptothrix*, *Crenothrix*, *Sphaerotilis* (or *Cladothrix*) and *Clonothrix*.

**Genus *Leptothrix*.**—*Leptothrix ochracea*, the best known species in this genus, is world-wide in its distribution. It is found in rivers, lakes, ponds and swamp waters which contain iron in chemical combination with organic matter (e.g., decaying plants).

The yellow- or reddish-colored slime found in the beds of iron-bearing waters nearly always owes its origin to the deposition of ferric hydroxide ("iron rust") in the outer, mucilaginous membranes of the long filaments formed by iron bacteria growing on the stream bottom. Living iron bacteria are, however, seldom found in such iron deposits since the incrustation of the membrane with iron occurs only in maturing filaments and eventually, since it oxidizes to ferric hydroxide and becomes hard and inelastic, interferes with their further reproduction. Young filaments may show little or no iron deposition and are of a grayish color and translucent appearance. These forms may be found as grayish flecks or specks in the water of slow streams or stagnant ponds in the spring and fall.

**Structure of *Leptothrix*.**—The younger threads are cylindrical and measure from 1.5 to 2 microns in diameter, somewhat thicker than ordinary bacteria. The longer threads may measure 300 microns in length.

Young threads in artificial cultures may be actively motile, although the presence of cilia has not been demonstrated. The

young threads sometimes adhere to the outside of older threads, giving the effect of branching.

When observed by ordinary methods, no internal structural details are readily observable in the threads. This has given rise to some difference of opinion as to whether the filaments are coenocytic cells like some mold hyphae, or whether they contain bacillus-like cells which can, when the sheaths become fully encrusted with iron, slip out and acquire a new sheath.

Threads that are covered with opaque iron hydroxide may be cleared by treatment with dilute acid. This will reveal the outer cell membrane. This membrane is covered by a very delicate, mucilaginous sheath or mantle in which the iron is deposited, but which is not easily demonstrated when full of iron hydroxide.

**Multiplication of *Leptothrix*.**—Multiplication may be by one or more of several methods. One of these is the method of fragmentation, common in such algae as *Nostoc*, although no true heterocysts are seen in iron bacteria. An alternate mode of multiplication is seen when small protuberances, conidia (or buds?) or "swarmers," form on the outside of the thread (Fig. 172). These elongate and eventually break off. Each forms a new thread. They sometimes appear in enormous numbers on the outside of *Leptothrix* threads. A thick crust of ferric hydroxide on old threads is obviously incompatible with such a mode of reproduction, so that it occurs only in young threads.

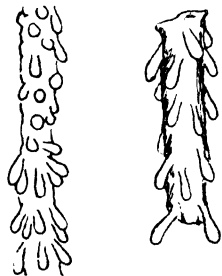


Fig. 172. — *Leptothrix ochracea*, showing method of reproduction by a process analogous to budding of yeasts. (Redrawn from Ellis.)

*Leptothrix* multiplies only in the presence of sunlight. From this fact one may infer the presence of some photosynthetic pigment although none has been demonstrated.

**Genus *Crenothrix*.**—The three genera to be described have an entirely different structure and mode of reproduction from *Leptothrix*. A description of one species, *Crenothrix polyspora*, will serve to illustrate features common to the other two genera, *Sphaerotilis* and *Clonothrix*. *Clonothrix* will not be discussed in detail.

**Multiplication of *Crenothrix*.**—*Crenothrix polyspora* starts life as a single, nearly spherical nonmotile cell which divides by fission and forms a chain, much as do streptococci, but differing in that the

cells elongate as they divide, so that the row shortly consists of bacillary forms. These exude a mucilaginous, tubular sheath of about 0.2 micron in thickness, which keeps the cells in a straight line, and glues one end of the filament to some object. This mucilaginous sheath soon hardens and becomes impregnated with iron hydroxide. The topmost cells are continuously being pushed out of the mouth of the sheath by the growing cells below. (Fig. 173.) In bodies of water containing much organic matter these filaments are seen as brownish "slime" or hairy covering on rocks.

The cells thus freed are called *macroconidia* and they start new colonies. Very often, in older plants, fission of the uppermost cells takes place in three planes before their liberation from the

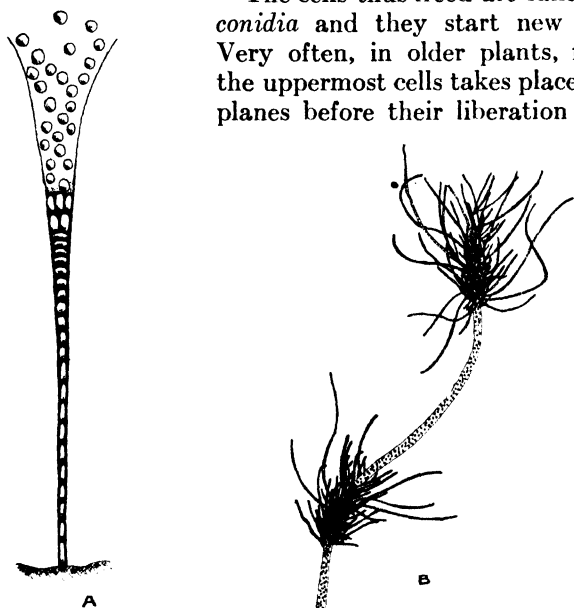


Fig. 173.—*Crenothrix polyspora*, showing, at A, method of reproduction by microcysts; B abnormal (?) reproduction. (Redrawn from Ellis.)

sheath. When this occurs the size of the free cells is much diminished and their number increased. Under these circumstances the cells are nearly spherical and are spoken of as *microconidia*. The increase in volume due to the formation of large numbers of microconidia within the sheath forces the latter to expand at the upper end so that a trumpet-like appearance is produced. This is a striking characteristic of *Crenothrix polyspora*.

Under certain conditions of nutrition, such as an abundance of iron-bearing organic matter, small numbers of competing or-

ganisms and plenty of oxygen and warmth, *Crenothrix* goes into a perfect orgy of reproduction. The cells in the sheath, from top to bottom, *all* start to multiply as microconidia at once and burst through the side, many remaining attached to the exterior of the parent plant and forming new threads, so that dozens or hundreds of brownish streamers may float from one thread (Fig. 173). Eventually the streamers break off and start the process anew. It is stated on good authority that the free microconidia also multiply as such, without waiting to go through the filament stage, producing reddish-colored, iron-containing zooglear masses of coccus-like bodies.

Under such conditions of growth *Crenothrix* may, in less than a week, change a large body of drinking water, initially sparkling and clear, to a lake of red-brown fluid which has been aptly referred to as a "horse-pond." The species name of *polyspora* is well merited.

When the mature plants die, under such conditions, they may decompose, with a resultant bad odor and taste that cause discouraging days in the "complaints" office of the head engineers of water purification plants. The saprophytic bacteria which cause decomposition of the dead *Crenothrix* increase in numbers so that the reports of the bacteriological laboratory to the engineer, on the numbers of bacteria present in the water, are also most discouraging, if not alarming. However, from the standpoint of disease causation, *Crenothrix* may be forgotten.

**Genus *Sphaerotilis*.**—The genus *Sphaerotilis* differs from any of the preceding by the presence, in the bacillary cells within the threads, of oil droplets and glycogen granules which can be demonstrated by appropriate means (Fig. 174). In this, *Sphaerotilis* has some resemblance to algae. The oil and glycogen act as reserve food. The genus is divided into two species, of which *dichotomus* is perhaps best known. It was formerly referred to as *Cladothrix dichotoma* and is still widely known by this name.

**Branching of *Sphaerotilis*.**—The species name *dichotomus* is derived from the peculiar habit of forming pseudodichotomous branches. These are best described by quoting Ellis: "In true dichotomy the growing point of a plant divides into two parts each of which grows into a branch, and one or both may again bifurcate in the same way. Under certain conditions this system of branching is *simulated* by *Cladothrix* (*Sphaerotilis*) *dichotomus* and a colony of this organism appears as is shown in Figure 175. This condition is brought about in the following way. Normally the cells

at the end of the thread slip out through the open mouth at the top of the sheath, but in addition some cells slip out *laterally*, making their way through the substance of the sheath. Usually these

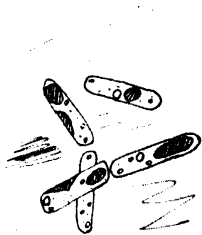


Fig. 174.—*Sphaerotilis* cells, showing oil globules and (dark-shaded bodies) glycogen granules ( $\times 900$ ).

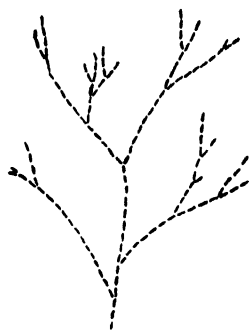


Fig. 175.—Diagram of dichotomous arrangement (false branching) of *Sphaerotilis dichotomus*. (Redrawn from Ellis.)

manage to get clear away, but occasionally they remain adherent to the sheath and there elongate to form a thread in attachment to the parent plant. This presents the appearance of a branch fixed to the thread, and when the process is repeated by several other cells

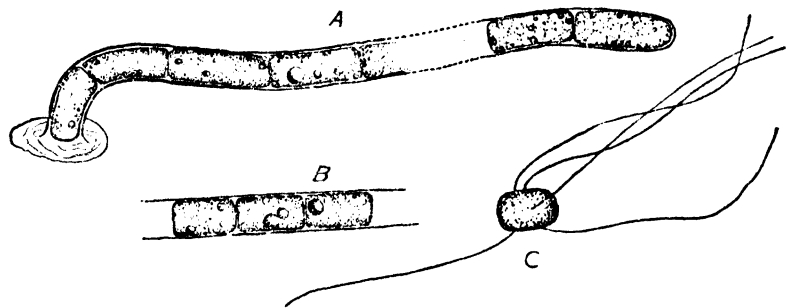


Fig. 176.—A, Attached strand of *Sphaerotilis*. Note the basal disc and the granular cytoplasm. B, Young cells, shorter than old slower growing cells C, Conidium, motile reproductive cell with irregularly attached flagella.

in different parts of the thread, and also by the cells of the 'branches,' which therefore in their turn will have 'branches' adherent to them, it can readily be imagined how a colony of threads is formed which has the general appearance shown in Figure 175. The branching is

thus seen to depend entirely on the adhesive capacity of the sheath, and it is clear that the connection between a filament and its branch is a purely fortuitous one."<sup>1</sup>

*Structure of Sphaerotilis.*—Each thread is a tubular sheath, as in *Crenothrix*, enclosing a single row of cells; but *Sphaerotilis* threads remain of a uniform diameter from end to end, since fission occurs in only one plane. The rod-shaped cells are about 2 microns wide and as much as 6 microns in length (Figs. 174 and 176). Iron is not always deposited in the sheath.



Fig. 177.—Free flocs of *Sphaerotilis* in aeration bottle arranged to simulate activated sludge. Air flow is stopped to show the ragged growths which sometimes clog the tanks. (Lackey and Wattie, U.S.P.H.S. Weekly Reports, Vol. 55.)

*Multiplication of Sphaerotilis.*—Multiplication is by means of fragmentation and by means of motile, ciliated macroconidia; the latter in *Crenothrix* as well as in *Sphaerotilis* are essentially a bacterial means of multiplication (fission).

When *Sphaerotilis* multiplies by fragmentation the free fragments of filament are motile by means of cilia, two or three at each end. These filaments are often spiral and resemble spirilla (not spirochetes!) in many respects. According to Ellis, spiral-shaped ciliated



cells also escape laterally from the filaments. These eventually become attached and form the usual type of filament. Microconidia do not appear. Cells in young filaments often escape laterally and start false branches. The empty spaces left by such escaping cells may function in the same manner as heterocysts in *Nostoc*.

Thus we find *Sphaerotilis* multiplying by motile or nonmotile thread fragments, straight or spiral, as well as by the escape of cells from the top of the tubes or from their sides, these cells also being either motile or nonmotile, straight or spiral.

*Sphaerotilis natans*.—This species is often found in activated sludge (see section on sewage disposal, page 450), and sometimes grows there to such an extent as to clog the tanks, causing a condition technically called "bulking" (Fig. 177).

**Systematic Relationships of Chlamydobacteriales.**—The relations of the Chlamydobacteriales to other classes of organisms are not entirely clear but in this connection we may consider certain outstanding facts. The order as a whole seems to be more bacterial than moldlike or alga-like. It is entirely aquatic while most molds are not. In the matter of thread formation, the Chlamydobacteriales resemble molds, but, as is true of the filaments of the *Actinomyces*, the threads are much tinier than any mold threads and are of the same order of magnitude as bacteria.

Further, although we speak of the daughter cells resulting from fission within the sheaths as *conidia*, they are really much more like ordinary bacterial daughter cells than the conidia borne on special organs of the molds. If the sheath were absent, they would probably not be called conidia.

Where branching is present, it is seen to be *false* branching due to adhesion of filaments to one another and not to an actual branching of individual cells. The resemblance to molds is again seen to be merely a superficial one.

Within the order, two very distinct types of organism are seen. The first is represented by *Leptothrix*. In this genus, resemblance to molds is seen in the apparently coenocytic, hyaline filaments. The absence of demonstrable nuclei is, however, more suggestive of the bacteria. The peculiar method of conidia formation in these genera is distinctly foreign to the Eubacteriales and suggests certain *Ascomycetes* (Yeasts; see Chapter 8), in which, in addition to multiplication by budding, thread formation occurs with fragmentation (*Oidium albicans*).

In the second type of iron bacteria (*Crenothrix*, *Sphaerotilis* and *Clonothrix*) bacteria-like cells are an outstanding feature. Indeed,

cells free from the sheath are hardly distinguishable from cocci or bacilli, since the bacillus-like cells frequently are motile by means of flagella.

The iron-bacteria are classed as "higher bacteria." Actually, if we consider the second type, we find them not so very much higher, but, as Ellis says, they may be regarded as bacteria which "have taken the first step toward what may be regarded the communal life." The more moldlike members of our first group may represent the nearest, but still deficient, approach which this order of plants could make toward becoming the more highly evolved molds or algae.

The view of the Chlamydobacteriales as forebears of the molds and algae in which sexual phenomena first become plainly visible, is particularly suggestive in light of a statement by Ellis. He says, "Under certain circumstances the rate of growth in size and the rate of division of bacteria do not run parallel. The rate of each is dependent on circumstances of which at present we have very little knowledge. If the rate of division is accelerated the individuals of successive generations become progressively smaller. Under certain circumstances the rate of division of *Crenothrix polyspora* . . . may so far exceed the rate of its growth that the individuals may be so far reduced in size that they are barely visible with the highest powers of the microscope." This increased cell division in low forms like *Crenothrix* results in minute individuals which form new plants directly. It might be regarded, however, as the forerunner of that increased cell division which, in higher forms, like *Ulothrix* and other algae, results in the development of cells (*microgametes*) so minute that they depend for nourishment and further growth on fusion with larger cells which have not divided to such an extent (*macrogametes*). In other words, this excessively rapid fission with decrease in size of cells may be the evolutionary precursor of male sexuality in plants and animals.

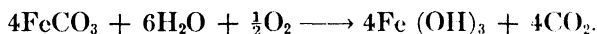
**Physiology of Chlamydobacteriales.**—A number of interesting points concerning the physiology of iron bacteria have been brought to light by artificial cultivation.

Cultures are usually studied in the natural water in which the organisms occur and are necessarily impure. The usual procedure in such cultivations is to add to the water small quantities of organic matter such as nutrient broth or hay infusion, along with iron salts. In this way increased multiplication of *Crenothrix*, *Sphaerotilis*, *Leptothrix* and other genera may be brought about. The medium may also be stiffened with agar. Such cultures are mixed.

Pure cultures of *Leptothrix ochracea* and of *Sphaerotilis* have been obtained aerobically on nutrient agar. *Sphaerotilis* grows also on neutral or slightly alkaline

nutrient gelatin which it slowly liquefies. *L. ochracea* has been cultivated by adding "manganese-peptone" and agar to the water in which it occurred. The colonies are rusty brown, round, and resemble those of ordinary bacteria in size and texture. Motile filaments can be demonstrated in them.

*Relation of Iron and Manganese to Iron Bacteria.*—These pure culture studies have brought out the fact that, while the iron bacteria have a peculiar affinity for iron-bearing organic matter, they may utilize only the *organic* and not the *iron-bearing* part of the molecules of such matter. Further, the iron released from the organic combination is deposited in the bacterial sheath where it is eventually oxidized to ferric hydroxide and finally forms so thick a crust as to end the reproductive functions of the organism. There is some difference of opinion as to whether or not the bacteria derive energy from the oxidation of the iron. Some believe that iron is oxidized spontaneously and that the bacteria derive no benefit from it. There is good evidence that some of the iron bacteria obtain the energy for chemosynthesis from the oxidation of ferrous compounds to ferric forms. This is true only of the autotrophic species. The reaction involved may be represented by the following:



The function of the iron in the life of those species not specifically requiring it as a source of energy is particularly mysterious when we learn that the plants may be cultivated, in pure culture, in the entire absence of this element or with only such traces as are necessary to the synthesis of the protoplasm. Aside from this requirement, they are said to be wholly independent of the metal, depending entirely on organic matter; yet they are found only in ferruginous waters and kill themselves by accumulating iron!

Another interesting fact is that manganese may be substituted for iron in these plants. If iron played an important role in their intimate physiology, it is hardly likely that manganese could replace it. But the two may appear mixed in the sheath in widely varying ratios. It would be quite appropriate to refer to the organisms as manganese bacteria. It seems to be the *organic* molecule which the heterotrophic iron bacteria utilize, regardless of whether iron or manganese is attached to it.

**Role of Iron Bacteria in Geology.**—A very interesting question in connection with the iron bacteria concerns their part in the formation of iron-bearing geological strata. A number of investigations have been made in this field. We have already seen how the ochre-colored deposits or slimy growths in the bottom of streams

and ponds of ferruginous water are often found to be composed of the threads of *Leptothrix ochracea* or some other species of Chlamydo-bacteriales. It is evident that the deposition of iron by such means is slow and that any very large deposits of ore due to bacteria must be so extremely old that the demonstration of bacteria in them would be a matter of great difficulty. In relatively recent deposits, however, such as the cakes of soft iron found beneath peat in bogs ("bog iron ore"), *Gallionella* and *Leptothrix* have been found in enormous numbers in various parts of the world. As for older geological formations, iron bacteria or iron fungi have been found in fossiliferous, ferruginous limestones. It is probable, however, that most large iron deposits result from purely physical or plutonic actions and are not in any way due to the action of bacteria. Nevertheless it is evident that, tiny though they may be and slow and primitive in their workmanship, these modest creatures may have contributed to man at least some portion of his proud "steel age" and to the history of the world.

### CAULOBACTERIALES<sup>3</sup>

These bacteria are almost entirely aquatic or marine in habitat, although some have been found in the soil. The living cells them-

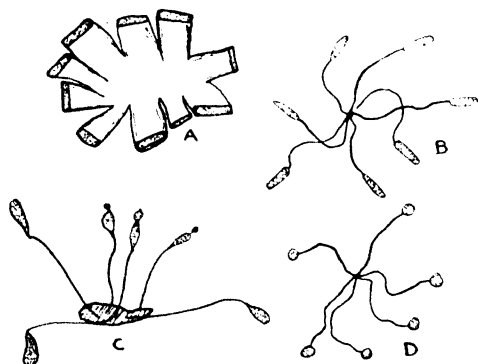


Fig. 178.--Various forms of stalked bacteria (Caulobacteriales). A, *Nerskia ramosa*, showing stalks formed by lateral secretion; bacteria (rods) at tips of stalks. B, *Caulobacter flagellatus*, showing stalks formed by terminal secretion. C and D, Other organisms, showing different modes of stalk formation. (Redrawn from Henrici.)

selves are, in general, much like true bacteria in size and shape and lack of visible internal structure, and have the general properties of the class Schizomycetes with respect to mode of multiplication.

nutrition and relation to sunlight. It might be logical to include them as a suborder of the Eubacteriales.

The peculiarity which sets them apart as a separate group is the fact that they form a secretion of gum or other matter *asymmetrically*, i.e., more is secreted at one side or end of the cell than at the other so that a stalk is formed as the result of, and consisting of, this secretion. Usually, the stalk is attached by a gluey "hold-fast" to some solid object in the water.

As the cells divide, the stalks become branched so that complex colonies may be formed, consisting of tangles or rosettes of filaments arising from common bases or centers, each stalk having a single living cell at its distal end (Fig. 178). The bacteria do not grow in strings or filaments of cells.

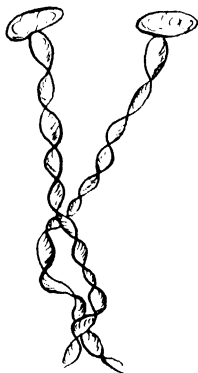


Fig. 179.—*Gallionella ferruginea*, showing bean-shaped bacterial cells and flat, twisted stalks.

The order Caulobacteriales is divided into four families, the Nevskiaceae, the Gallionellaceae, the Caulobacteriaceae, and the Pasteuriaceae.

**Gallionella Ferruginea.**—This species, formerly included with the Chlamydobacteriales (so-called "iron bacteria") because it formed a stalk of ferric hydroxide, is now classed with the Caulobacteriales because it has been found that the organism is neither filamentous nor sheath-forming, but does spin a stalk. *Gallionella ferruginea* (formerly known as *Didymohelix ferruginea*) is the best known species of the order.

This organism is found in nature as widely distributed as *Leptothrix* of the Chlamydobacteriales, which it much resembles. Owing to its ability to grow well in the absence of sunlight, it can multiply in water pipes, whereas *Leptothrix*, requiring sunlight, is held in check to a great extent in such situations. In water conduits made of iron, *Gallionella* causes extensive deposits and incrustations of iron which may eventually occlude the pipes.

Structurally, the stalks of *Gallionella* are almost indistinguishable from those of *Leptothrix*. They have, however, a rather flat form and the remarkable habit of twisting spirally upon themselves, so that they resemble a loosely coiled hairpin or rubber band (Fig. 179). Iron is deposited in this stalk.

The twisting habit renders identification of *Gallionella* easy, since no other organism of similar character is known to twist in just this

way. The loops in the coils are traversed by the mucilaginous secretion and when, in old plants, these become fully encrusted with opaque iron hydroxide, the stalk of the organism takes on the appearance of a row of beads.

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2. Lackey, J. B., and Wattie, E. Studies on Sewage Purification. U. S. Pub. Health Service, Weekly Reports, 1940, 55:975.
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#### CHAPTER 24

### THE SULFUR BACTERIA

IN THE SECTION on Nitrobacteriaceae there will be described, under the name *Thiobacillus*, autotrophic bacteria capable of oxidizing elementary sulfur and various sulfur compounds like thio-sulfate, to sulfuric acid. These bacteria, as will be shown, resemble the true bacteria (order Eubacteriales) in all other respects. The generic name *Thiobacillus*, applied to these true bacteria, must be carefully distinguished by the student from the somewhat similar names of the entirely separate order of Thiobacteriales which we are about to study.

This order is made up of bacteria which, with a few exceptions, are much like Eubacteriales but which possess one or both of two distinctive properties. The first of these is the possession of a pigment system containing red *bacteriopurpurin* and green, photosynthetic *bacteriochlorophyll*. The species containing this pigment system are grouped in the family Rhodobacteriaceae. The second distinctive feature is the storage of elemental sulfur *inside* the cells in the form of large globules. The species having this feature, but not the pigment system, are grouped in the families Beggiatoaceae (filamentous forms) and Achromatiaceae (nonfilamentous forms).

The family Rhodobacteriaceae is divided into two groups depending on whether or not sulfur granules are stored in the cells. Species storing the sulfur are grouped as Thiorhodaceae, those not

storing granules are grouped as Athiorhodaceae. These relationships are shown in the following outline:

Thiobacteriales:—

Photosynthetic pigments

Thiorhodaceae (store sulfur)

Athiorhodaceae (do not store sulfur)

No pigments

Beggiatoaceae (filamentous)

Achromatiaceae (not filamentous)

It is an interesting question whether the Athiorhodaceae should be removed from the order and scattered among the true bacteria according to morphology because they do not store sulfur, or included because they have photosynthetic pigment and the non-pigmented species excluded from the order instead.

So far as is known, the Thiobacteriales have no medical or industrial importance, although the industrial aspect of the group may some day be developed. However, the action of sulfur bacteria as scavengers and as utilizers of malodorous and poisonous sulfur compounds ( $H_2S$ ) resulting from organic decomposition in sewage and boggy places, and as producers of sulfates, must be kept in mind. The production of sulfates is highly important, since sulfur is available to the higher plants mainly in this form. A sulfur cycle is as important to life as is a nitrogen or carbon cycle. (See section on soil bacteria, page 420.)

**Utilization of Sulfur by Thiobacteriales.**—Thiobacteriales, as the name implies, are active in the metabolism of sulfur compounds, especially hydrogen sulfide, but they differ from the true bacteria of the genus *Thiobacillus* in several important respects. When the latter oxidize hydrogen sulfide, elemental sulfur is set free *into the surrounding medium* where, in turn, it is later oxidized to sulfuric acid.<sup>1</sup>

The bacteria which have been included in the order Thiobacteriales also metabolize hydrogen sulfide and other simple sulfur compounds. The photosynthetic species use it as a hydrogen donor in the same manner that green plants use water in photosynthesis. With the exception of a few species, these organisms also liberate free sulfur. However, as noted above, many species store the sulfur inside the cells in the form of globules and granules (Fig. 180) where it appears to function as a reserve food substance much as does starch or glycogen in plant and animal cells, and is later oxidized to sulfuric acid which immediately forms sulfates.<sup>2</sup>

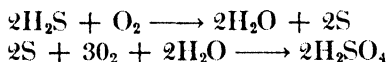
These sulfur granules are a prominent feature of most of the

Thiobacteriales and constitute one of the chief reasons for grouping together a number of rather dissimilar, bacterium-like plants which have this characteristic in common. As noted above many of the Thiobacteriales also contain red *bacteriopurpurin* and green *bacteriochlorophyll*, the latter a pigment closely related to true chlorophyll and enabling those species which contain it to reduce carbon dioxide by means of light energy (photosynthesis). Bacteriochlorophyll is effective in light of longer wave lengths, red and infra-red, than that activating true chlorophyll.<sup>3</sup> None of the Thiobacteriales produces spores or conidia.



Fig. 180.—*Beggiatoa alba*. Portion of a filament containing globules of sulfur. (Henrici, "The Biology of Bacteria," D. C. Heath and Company, publishers.)

**The Colorless Sulfur Bacteria.**—The nonpigmented or white sulfur bacteria utilize hydrogen sulfide and sulfur as sources of energy for the assimilation of carbon dioxide and the synthesis of their cell substance, probably according to the equations



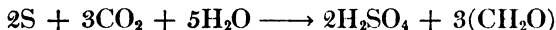
the acid combining with chlorides, metals, etc., to form sulfates and other stable compounds. They are autotrophic and aerobic, requiring free oxygen as indicated in the equation given.

The commonest species of this type is *Beggiatoa alba*. *Beggiatoa* are filamentous and show creeping and waving movements suggestive of the alga *Oscillatoria*. Another species, *Thiothrix nivea*, forms a sheath about filaments of rod-shaped cells, and is attached to a solid base, properties suggestive of some of the iron bacteria.

**The Pigmented Sulfur Bacteria.**—The pigmented bacteria (Rhodobacteriaceae) of this order, which are of two kinds, green and red-purple, utilize hydrogen sulfide, but in an entirely different manner from the nonpigmented species. Most are anaerobic (especially Thiorhodaceae). Because of the presence of their photosynthetic pigment these organisms under *anaerobic* conditions ob-

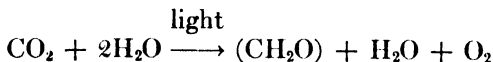


tain the energy necessary for the assimilation of carbon dioxide from sunlight. Good growth will not occur in the dark as a rule. Some, however, can grow in the dark as well as in the light. But photosynthesis does not occur and hydrogen is not transferred to carbon dioxide but to *oxygen*, necessitating *aerobic* conditions. The *metabolism, in other words, becomes like that of other aerobic bacteria of the order Eubacteriales*. The photosynthetic feature seems merely to be added to the ordinary system of metabolism in these bacteria. In the sunlight under *anaerobic* conditions hydrogen sulfide (or organic substrate or molecular hydrogen, see below) is not used as a source of energy, but as a hydrogen donor in the photosynthetic reduction of the carbon dioxide incident to its incorporation as part of the cell. This changes hydrogen sulfide to sulfur and represents oxidation. The released sulfur may or may not be stored in the cell as free sulfur granules, depending on the species. Free oxygen is not given off in bacterial photosynthesis. The red and purple species of Thiorhodaceae retain the sulfur as intracellular granules which are eventually oxidized to sulfuric acid.

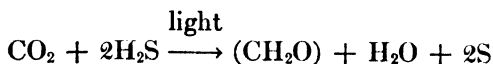


The Athiorhodaceae, as the name implies, do not store the sulfur but liberate it into the surrounding medium. Both types use hydrogen sulfide in photosynthesis.

The type of reaction developed by van Niel<sup>3</sup> for photosynthesis in general may be adapted to bacterial photosynthesis as a special case in which hydrogen sulfide substitutes for water. In green plant photosynthesis the hydrogen is presumably removed from water under the influence of sunlight and is then incorporated with carbon dioxide in some sort of organic compound generally represented in the reaction formulas as  $CH_2O$ . A simplified view of this reaction is as follows:

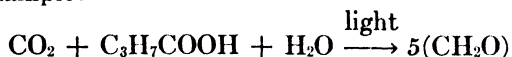


The same reaction involving hydrogen sulfide may proceed as follows:

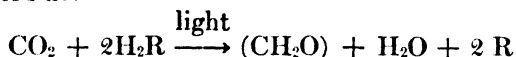


Most of the purple sulfur bacteria (Thiorhodaceae in pure culture only) are also able to remove hydrogen from certain organic

compounds. The same general reaction holds however; the hydrogen sulfide is replaced by the organic hydrogen donor in question. For example:



Such organisms do not accumulate sulfur granules for obvious reasons. Some of these bacteria can use molecular hydrogen directly. A general formula, representing the type photosynthetic reaction, may be stated as:



where R indicates the remainder of the molecule from which the hydrogen is abstracted.

**Habitat of Sulfur Bacteria.**—Sulfur bacteria are found in water and swampy soils all over the world, especially wherever saprophytic organisms are actively decomposing organic matter with the production of hydrogen sulfide, which they metabolize. The growth of *sulfate-reducing* organisms is also important in producing the hydrogen sulfide necessary to the sulfur bacteria. Some species, especially of *Athiorhodaceae*, can also oxidize thiosulfate as energy source and use molecular hydrogen in photosynthesis. They may be observed in such places as decomposing sea weed, in rock pools, stagnant woodland pools and sewage. The colored varieties (*Rhodobacteriaceae*) develop well only when exposed to light. In certain polluted bays they multiply to such an extent as to cause the entire body of water to look red or purple. Certain species of sulfur bacteria grow in hot sulfur springs (*Thiorhodaceae*; autotrophic), and others around sulfur mines. Sulfur bacteria may also be responsible for some of the large deposits of this element, which are of great commercial value. Occasionally certain of the colorless sulfur bacteria grow in water supplies, much as do the iron bacteria, and obstruct pipes and are the source of foul odors and tastes due to their decomposition by other bacteria when they die.

**Morphological Characters of Thiobacteriales.**—As regards morphology, the sulfur bacteria occur in all of the forms found in the Eubacteriales. Indeed, many of those classified in the group of Thiobacteriales might be included with true bacteria were it not for their intracellular storage of sulfur or their photosynthetic pigment. As van Niel states: "If, under certain conditions, one of the non-sulfur purple bacteria [*Athiorhodaceae*] would fail to produce its prominent pigment system, it would thereby become indis-

tinguishable from a typical *Pseudomonas*, *Vibrio* or *Spirillum* species."<sup>4</sup> The names of many genera are derived from those of true bacteria by prefixing *Thio* (sulfur); for example, *Thiovibrio*, *Thiospirillum*, *Thiococcus*, *Thiobacillus*, etc. Long filamentous forms occur in some groups. True branching, such as is found in the Actinomycetales is, however, absent among the Thiobacteriales. Spores are absent also. Size varies from extremely minute cocci to elongated forms having a length of nearly 0.2 mm. (200 microns).

*Forms of Beggiatoaceae.*—In the development of some species of sulfur bacteria, one or more changes in form or arrangement of the

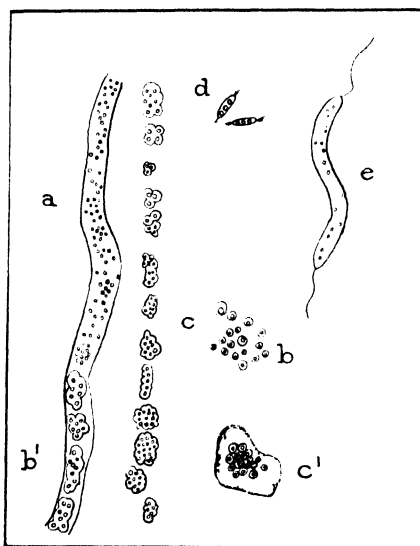


Fig. 181.—*Beggiatoa alba*, showing pleomorphism. (Redrawn from Ellis.)

cells are described. As an illustration let us consider a common species (*Beggiatoa alba*) belonging to the group of colorless, filament-forming sulfur bacteria. This organism is found in sulfur springs and in waters containing putrefying organic matter.

On the beds of sewage-polluted streams *Beggiatoa alba* forms a slimy, grayish growth composed of threads sometimes 50 to 100 microns long and 2 to 6 microns in diameter. These threads show a slow, waving motion like that of *Oscillatoria*. The filaments, like those of the iron bacteria *Leptothrix* and *Spirophyllum*, are non-septate, and a slimy sheath is said by some to form, as in *Crenothrix*,

but probably does not. The threads contain numerous, oil-like droplets of free sulfur. Reproduction of *B. alba* is by fragmentation of the filaments (Fig. 181, *a*). Fission also occurs as in true bacteria. When fragmentation occurs, the fragments may cling together for a time (Fig. 181, *b'*). These fragments may further divide into spherical, motile, coccus-like cells (Fig. 181, *b*), or they may coalesce into zooglear masses (Fig. 181, *c* and *c'*). Motile, rod-shaped cells are also formed in this stage (Fig. 181, *d*). It is said also that portions of the filament often assume the form of a rigid spirillum with cilia at both ends (Fig. 181, *e*). These various forms probably eventually elongate to form the threads characteristic of the *Beggiatoaceae* (Fig. 181). This general type of pleomorphic development is seen in a number of species of sulfur bacteria. However, it is possible that some of the "pleomorphic forms" are seen in impure cultures and are not part of the cycle of *B. alba*. The study of pure cultures of these organisms has been very difficult.

*Forms of Achromatiaceae.*—Most species of the colorless, *nonfilament-forming* family of Achromatiaceae occur in sulfur springs, sea mud and brine ditches. A good example is seen in *Thiophysa volutans*. This appears *only* as globular or ovoid cells (Fig. 182), never forming filaments. The organisms move slowly in a forward and rotary manner without the aid of demonstrable cilia. The cells are usually large, 9 to 33 microns in diameter, and from 15 to 85 microns long. Multiplication seems to be solely by fission. Various unicellular ovoid, ellipsoidal or round species are known in the four genera of this family, some with cilia, and of varying degrees of complexity of structure and activity.

*Forms of Rhodobacteriaceae.*—In the pigmented, *nonfilament-forming* family of Rhodobacteriaceae the cells are elongated, ellipsoidal, ovoid or coccus-like. As noted above, many are exactly like true bacteria except for their pigment. Many of them form aggregations in masses of slime (Fig. 183). This slime eventually liberates the cells which may then be motile or nonmotile. Some species are rod-shaped or spirillum-like and some vary in form. All contain the pigments *bacteriopurpurin*, and *bacteriochlorophyll* which acts in photosynthesis.

An interesting feature of one genus of this family, namely, *Rhodocapsa*, is the presence of *aerosomes*. These are irregular, red-



Fig. 182.—*Thiophysa volutans* ( $\times 900$ ). Note the sulfur granules. (Redrawn from Ellis.)

dish-colored bodies which refract light and are said to help suspend the cell in water (Fig. 184). Aerosomes also occur in some of the blue-green algae and, together with the slime and the photosynthetic pigment, suggest very strongly that here we may find a transitional stage between these "higher" bacterial forms and the lower forms of algae.



Fig. 183.—*Rhodotheca pendens*, showing zoogloeal mass ( $\times 900$ ). (Redrawn from Ellis.)

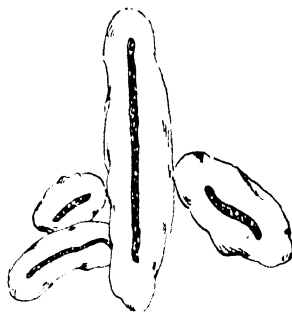


Fig. 184.—*Rhodocapsa suspensa*, showing aerosomes and zoogloeal material ( $\times 900$ ). (Redrawn from Ellis.)

Other thought-stimulating genera of the pigmented Rhodobacteriaceae are *Amoebobacter* and *Thiodictyon*. In the former the cells are coccoid. They are connected by threads of contractile plasma and they exude a slimy, gelatinous matrix enclosing the whole. The plasma threads connecting the cells pull the family up into heaplike masses, or allow the cells to spread apart (Fig. 185),

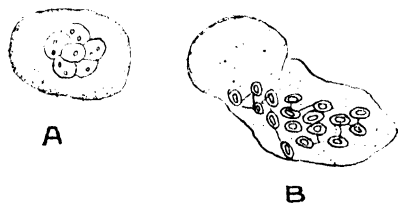


Fig. 185.—*Amoebobacter roseus* ( $\times 900$ ). *A* Contracted; *B* extended. (Redrawn from Ellis.)



Fig. 186.—*Thiodictyon elegans* ( $\times 900$ ). (Redrawn from Ellis.)

strongly suggestive of an ameba or of the "swarm" stage of an order of bacteria which we have previously studied, the Myxobacteriales, in which whole families of rods in slime move about from place to place.

*Thiodictyon* forms a network of spindle-shaped cells united at the

ends so that a net is formed which is very reminiscent of the alga *Hydrodictyon* (Fig. 186).

**Cultivation of Sulfur Bacteria.**—The cultivation of sulfur bacteria in bottles of polluted water is not difficult. The photosynthetic types must be exposed to sunlight and air must be excluded. *Pure* cultures have been obtained of certain species of *Beggiatoa* and of some of the Rhodobacteriaceae, the latter largely through the researches of van Niel. As stated by this author: "It is hoped that the publication of the results may make these bacteria more generally known to microbiologists, and thus lead to more intensive studies of the many fascinating problems which they still present."<sup>4</sup> Cultures of *Beggiatoa* are started by placing a small flake of the grayish growth from a bog, or other source, in distilled water and washing off extraneous matter as far as possible, then transferring to a flask containing a solution having the following composition:

$\text{CaH}_2(\text{CO}_3)_2$ .....	0.34 gm.
$\text{MgH}_2(\text{CO}_3)_2$ .....	0.27 gm.
$\text{CaSO}_4$ .....	0.31 gm.
$\text{MgSO}_4$ .....	0.51 gm.
$\text{Na}_2\text{SO}_4$ .....	0.21 gm.
$\text{Ca}_3(\text{PO}_4)_2$ .....	0.02 gm.
$\text{KCl}$ .....	0.01 gm.
$\text{K}_2\text{S}$ .....	0.01 gm.
$\text{FeS}$ .....	0.01 gm.
$\text{CaS}$ .....	0.01 gm.
$(\text{NH}_4)_2\text{SO}_4$ .....	0.04 gm.
$\text{H}_2\text{O}$ .....	100.00 gm.

The air space in the flask is filled with an atmosphere of oxygen, hydrogen sulfide and carbon dioxide. In two weeks at 30° C. threads are found multiplying freely and the culture is washed repeatedly with sterile distilled water. Contaminating organisms must be washed out so completely that the addition of peptone to the wash water results in no growth of ordinary, saprophytic bacteria.

*Raw* cultures of sulfur bacteria are obtained in much the same manner as the raw cultures of iron bacteria. The material containing them is *enriched* by the addition of water containing hay, eggs or peptone or, for marine forms, sea water containing decomposing fish or crabs or seaweed. A limited supply of oxygen is desirable and may be obtained by placing the materials in a narrow-mouthed bottle, filled to the neck but left unstoppered. Sea water-peptone agar may yield pure colonies of some sulfur bacteria when inoculated from such primary cultures. For more exact details of pure

culture methods the student is referred to the monograph by van Niel.<sup>4</sup>

**Relations of Light to Thiobacteriales.**—Some very interesting observations have been made on the effects of light on sulfur bacteria. Some of these organisms are among the most sensitive of all organisms to the presence or absence of light. If a spectrum be thrown on a culture of Rhodobacteriaceae the organisms will congregate most thickly in the infra-red portions and to a lesser extent in other bands. It is of interest to note that the infra-red is also the region in which the bacteria absorb energy to the greatest extent. It is also of interest that the infra-red rays were probably the first to pierce the murky atmosphere of the primitive earth and these bacteria may represent descendants of the first organisms able to make use of photosynthetic processes.

Light also markedly affects the motility of colored sulfur bacteria. When light is withdrawn for too long a time they lose their motility permanently. If steadily exposed to intense light they also lose motility, but will maintain it if the intensity of the light be varied from time to time.

If certain motile, colored sulfur bacteria come to a shaded spot they stop, reverse their motion, wait a few seconds, and then go on again more slowly. This is spoken of as a "shock movement." Similar "shock movements" have been described when these organisms pass from light of one color to that of another color.

*Thiospirillum jenense* (an organism of the family Rhodobacteriaceae and much like the genus *Spirillum* in the Eubacteriales but containing sulfur granules) has a single, polar flagellum. By arranging a patch of shadow so as to move over a swimming cell of *T. jenense*, it has been shown that there is, in this species, a definite point where sensitivity to light is localized. If the shadow overtakes the organism from "behind," a "shock movement" occurs as soon as the point of insertion of the cilium is covered. If the shadow approaches the cell from the direction in which it is traveling, no "shock movement" occurs until nearly the whole spirillum is in the dark; in other words, until the sensitive spot near the base of the cilium is shaded. This localization of sensitivity to light recalls vividly the red "eye spot" of *Euglena viridis*.

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## CHAPTER 25

### BACTERIA IN SOIL

#### THE NITROGEN AND SULFUR CYCLES

HUMAN BEINGS are prone to give much attention to things that disturb them, hence the uninitiated are apt to think of bacteria only as the cause of disease and to overlook the fact that of the hundreds of known species, only a few are pathogenic while the rest are either harmless commensals, or are very valuable in industry or agriculture. Indeed, man is wholly dependent upon microorganisms of the soil, some of which prepare nitrogen, phosphorus and sulfur in the soil or atmosphere by chemical combinations so that they may be utilized by more complex plants and animals; others decompose complex nitrogenous, sulfur-bearing and phosphorus-containing molecules of plant and animal tissues and wastes, setting free the elements, which again are returned to the soil or atmosphere or immediately utilized. Many elements, like nitrogen, phosphorus and sulfur, constantly undergo this cyclical alternation between the organic and inorganic systems, bacteria of certain sorts playing an important role in various stages of these cycles. We shall discuss some of these cycles in this chapter.

Many soil microorganisms, while not so absolutely essential for human existence as those involved in cycles of the essential elements, are nevertheless highly necessary to man's prosperity and health, to say nothing of his comfort, because of their powers of decomposing dead organic matter, killing many pathogenic species, and increasing soil fertility. Not only are soil microorganisms of great importance from an immediately practical viewpoint, but by studying them we learn much of general biological interest and importance.



Most of the organisms described in this book are indigenous to the soil, so that we cannot cover the entire subject of "soil bacteriology" in this single chapter. However, we may begin by outlining general methods for investigating the bacterial flora of soil, and then describe some of the species most important to the actual existence of human life, the nitrogen bacteria and the nitrogen cycle, some of the sulfur bacteria and the sulfur cycle, bacteria of importance in the phosphorus cycle and related species.

**Bacteriological Examination of Soil.**—Since fertile soil contains anaerobic organisms, strict aerobes, thermophils, autotrophs, heterotrophs, and other varieties having specialized growth requirements, no single method can be given for cultivating or enumerating soil bacteria in general.<sup>1</sup> Three procedures are available for crude studies yielding approximate results. For exact results the methods of *selective enrichment* (providing a medium which favors one species but not others) or *selective bacteriostasis* (providing a medium which permits growth of a desired species but inhibits others) must be used. Applications of selective methods are described in detail elsewhere, *e.g.*, enteric group.

**Plating Methods.**—The plating method is applicable to the enumeration and isolation of bacteria in any substance, such as soil, water, milk, blood, feces, etc., and suitable modifications are made to meet the cultural requirements of the bacteria likely to be found in the substance examined. In examining soil a small weighed sample (about 1 gm.) is thoroughly shaken up with a measured volume (say 5 cc.) of sterile water. A series of dilutions of the water, which now contains many free bacteria and fine particles of soil to which others adhere, is prepared and 1 cc. of each dilution is placed in a sterile Petri dish. Each dish receives about 15 cc. of melted extract or other nutrient agar, cooled to about 45° C. This is thoroughly mixed with the diluted sample by gently tilting and rotating the plate.

After incubation, which may last from twenty-four hours to two weeks, preferably at about 25° C. since many soil bacteria grow well at this temperature, the colonies are counted and the number of organisms per gram of soil computed from the dilution, the weight of the sample, and the volume of wash water.

Obviously the method is inexact since anaerobes and thermophils will not grow under the conditions provided, neither will obligate autotrophs nor species having other special requirements. For each of these, the medium used and the conditions of temperature, pH, etc., must be appropriately adjusted, *i.e.*, selective cultivation must

be practiced. However, the plate method is of great practical value in many soil studies.

*Dilution Methods.*—These are special applications of the method of selective cultivation or enrichment. Dilutions of the soil sample are prepared as for plating but portions of each are placed in special media. For example a series of dilutions is placed in tubes or plates containing cellulose as the only source of carbon. Only cellulose digesters can grow. If media are used containing no nitrogen and only inorganic substances, only nitrogen-fixing, autotrophic bacteria can grow. From the dilutions in which growth occurs, the approximate numbers of the types for which provision has been made can be stated. Other types are not counted.

*Microscopic Examination.*—By making stained smears of soil and examining them with the microscope we may count various morphological types of bacteria and other microorganisms. The principal inaccuracy in this method is that dead as well as living cells are counted. Another arises from the difficulty in staining some species and still another from errors in differentiating minute soil and other inert particles from bacteria, as well as morphologically similar species of bacteria from each other.

A weighed soil sample is suspended in 5 to 10 times its weight of very dilute gelatin, agar or egg albumen. The viscous substances serve to glue the bacteria to the slide. A measured amount (0.01 cc.) of the suspension is spread over a given area of the slide (1 sq. cm.) dried, and stained with carbol fuchsin, methylene blue or other dye. After washing and drying, the bacteria are counted. For the method of computing the numbers per gram of sample, see the section on direct microscopic examination (Breed count) of milk (page 562).

The methods mentioned above, while inexact, have proven extremely useful and fruitful in soil microbiology. It has been found that bacteria inhabit only the upper layer of soil, seldom occurring in any large numbers below the level of 2 feet from the surface. Good fertile loam may contain anywhere from 100,000 to 500,000,000 per gram.

Among these soil bacteria we find nearly every form of bacterial morphology and almost every type of metabolism known to the class of fission fungi. Among the important groups are those comprising the families Nitrobacteriaceae, Rhizobiaceae and Azotobacteriaceae. In order to appreciate fully the important functions of these bacteria, it is desirable to consider especially their remarkable metabolic habits. In general, they are autotrophic, and may

either be aerobic or anaerobic with regard to oxygen. Some are facultative in both respects.

### THE NITROGEN BACTERIA

The nitrogen bacteria are so called because they are concerned in the oxidation of ammonia and nitrites to nitrates, an important phase in the nitrogen cycle, as we shall see. The nitrogen bacteria are autotrophic, and utilize ammonia or nitrites as sources of energy, depending on the species involved. The change from ammonia to nitrates is stepwise and may be stated as follows:

1. Ammonium salts to  $\text{HNO}_2$ . Genera *Nitrosomonas* and *Nitrosococcus*
2.  $\text{HNO}_2$  to  $\text{HNO}_3$ . Genus *Nitrobacter*

Probably some of the most primitive species of bacteria known are grouped in the tribe Nitrobacteriaceae. The three genera referred to above are spoken of as nitrifying bacteria because together they are capable of oxidizing ammonia, possibly formed in the atmosphere by lightning discharges, to nitrites and nitrates. Ammonia

from any other source, as decay of protein, is likewise nitrified.



Fig. 187.—*Nitrobacter* ( $\times$  about 900).

Fig. 188.—*Nitrosococcus* ( $\times$  about 900).

What may be supposed to be among the most primitive forms of the Nitrobacteriaceae (the genus *Nitrosococcus* and the genus *Nitrosomonas*) are capable of oxidizing the ammonia only to nitrites, a process called *nitrosification*. Many higher plants cannot utilize nitrites as their only source of nitrogen, and hence their best development is dependent on the presence of the *Nitrobacter* which oxidize the nitrites to nitrates (*nitrification*).

These three genera of bacteria form a rather heterogeneous group of gram-negative and possibly gram-positive rods and cocci (Figs. 187 and 188). None of them forms spores. The *Nitrosomonas* are motile rods, the *Nitrobacter* are nonmotile rods. They are all aerobic and thrive in warm, moist, slightly alkaline (pH 7-8) soils. They

also occur in rivers, streams, etc., and are world-wide in distribution. As judged by test-tube experiments, all live best in the entire absence of organic matter. Their energy sources are very different from those made use of by higher forms of life like protozoa, pathogenic bacteria and insects, which utilize only complex organic substances derived from living or dead plants or animals. Consider what is burned ( $\text{NH}_4\text{OH}$  and  $\text{HNO}_2$ ) by these autotrophs, and what is the ash ( $\text{HNO}_3$ )! The other elements necessary for the synthesis of the protoplasm of Nitrobacteriaceae are obtained from the inorganic salts commonly present in soil or rocks. The *Nitrosomonas* will grow in a solution containing

$(\text{NH}_4)_2\text{SO}_4$ .....	2.0 gm.
$\text{K}_2\text{HPO}_4$ .....	1.0 gm.
$\text{MgSO}_4$ .....	0.5 gm.
$\text{FeSO}_4$ .....	0.4 gm.
$\text{NaCl}$ .....	0.4 gm.
$\text{H}_2\text{O}$ .....	1000.0 cc.

A lump of marble is added to prevent acid accumulation.

The nitrifying organisms may be cultivated in solutions such as the preceding by substituting sodium nitrite for the ammonium sulfate.

Thus we find one of the mechanisms by which inert atmospheric nitrogen is made available to complex plants and animals and to man. It may be combined with hydrogen by lightning to form ammonia. It may also be released, in the form of ammonia from protein by bacteria and other fungi which decompose proteins. Through the action of the nitrogen bacteria the nitrogen in the ammonia is made available to higher plants. To these bacteria, in the last analysis, the proudest nations owe their wealth and power. We see in this mechanism the beginning of a cyclical interchange of nitrogen, which is discussed later as the *nitrogen cycle*.

#### FAMILIES AZOTOBACTERIACEAE AND RHIZOBIACEAE

From the standpoint of the nitrogen cycle and the fertility of soil due to increases in the available nitrogen content by bacterial action, the organisms of the genus *Azotobacter* and genus *Rhizobium* must be regarded as among the most important. The organisms of the two genera named have the remarkable property of being able to take the inert, uncombined nitrogen of the atmosphere and combine it with other elements to form compounds from which, upon their decomposition, it is liberated in a form available to farm crops either directly or through further bacterial action. This proc-

ess of synthesizing free nitrogen into compounds is called "nitrogen fixation" and constitutes an important factor in the "nitrogen cycle."

Fixation of nitrogen as accomplished by the aerobic *Azotobacter* (and certain species of anaerobes) is said to be *nonsymbiotic*, in contrast with the mechanism by which the *Rhizobium* species fix nitrogen in *symbiotic* combination with certain plants. The aerobic, nonsymbiotic fixation of nitrogen will be discussed first.

### Genus *Azotobacter*. Nonsymbiotic Aerobic Nitrogen Fixation.

—The *Azotobacter* live in well aerated, slightly alkaline, arable soils and are strict aerobes. Some are motile, others non-motile. They are large, non-spore-forming, usually rod-shaped bacteria, but often grow in oval or yeastlike forms (Fig. 189). They oxidize carbohydrates and other non-nitrogenous organic compounds as a source of carbon and energy and can obtain their nitrogen *directly from the air*. In the soil, the carbohydrates needed for their energy are probably derived

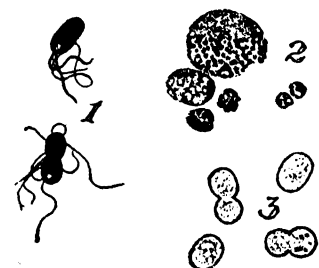


Fig. 189.—Nonsymbiotic nitrogen-fixing bacteria. 1, *Azotobacter agile*; 2, *Azotobacter chroococcum*, degenerate forms; 3, *Azotobacter agile*. (After Beijerinck.)

ordinarily from the decomposition of the cellulose of higher plants by acid-producing and cellulose-decomposing soil bacteria. It has been found that carbohydrates added to the soil in the form of molasses, starch wastes and the like act as fertilizer. The fertility results in part from the accumulation of nitrogen in the soil through the growth of the *Azotobacter*, which use the carbohydrates as energy sources (Fig. 190), and combine the nitrogen in their cell-protoplasm, whence it is released on the death of the bacteria. The *Azotobacter* grow readily in such solutions as the following:

H <sub>2</sub> O .....	1000.0	cc.
Mannitol (source of energy) .....	15.0	gm.
K <sub>2</sub> HPO <sub>4</sub> .....	0.2	gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	0.2	gm.
CaCl <sub>2</sub> .....	0.02	gm.
FeCl <sub>3</sub> (10 percent aq. sol.) .....	0.05	cc.
Molybdenum salt .....	Trace	
Adjust to pH 7.2. For solid medium, add 15 gm. of agar before adjusting the pH.		

Note the absence of nitrogen source.

A solution of this kind will, when inoculated with soil, yield a good growth of *Azotobacter*, and they may be isolated from it on agar plates.

The importance of molybdenum should be pointed out. In the absence of this element in minute amounts nitrogen fixation will not occur. It has been shown also<sup>1a</sup> that most species of *Azotobacter* will use ammonia nitrogen in preference to atmospheric nitrogen. They can also utilize nitrates, nitrites, urea and asparagine, and will do so to some extent even if atmospheric nitrogen and molybdenum are present. However, under most soil conditions urea is rarely present in a significant amount while ammonia, though constantly



Fig. 190.—Buckwheat and oats fertilized with sugar, after A. Koch. Pots 101 and 166 were not treated; 117 and 162 received sugar. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Book Co., Inc., publishers.)

being formed by decomposition, is quickly removed by bacterial oxidation or assimilation by higher plants. Nitrates are not often present in a sufficient concentration to decrease fixation of atmospheric nitrogen markedly.

**Nonsymbiotic Anaerobic Nitrogen Fixation.**—Nitrogen fixation is carried on in swampy soils by at least one species of bacterium belonging to the group of gram-positive, spore-forming, strictly anaerobic, heterotrophic, saprophytic rods called *Clostridium*. They are common in boggy, acid soils where the *Azotobacter* cannot grow. This organism has no relation to the Nitrobacteriaceae beyond the

fact that it uses atmospheric nitrogen to build its protoplasm and thus fixes free atmospheric nitrogen in the soil. It is mentioned here solely because of this. The species in question was described about

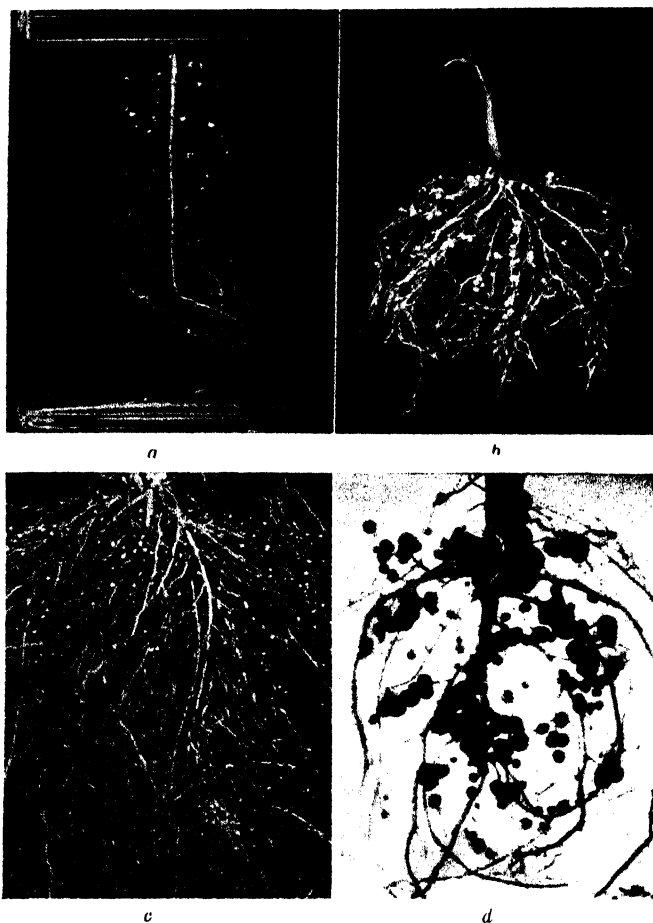


Fig. 191.—Roots (with nodules) of (a) young alfalfa, (b) young pea, (c) mature red clover, (d) mature soy bean. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Co., Inc., publishers.)

1902 by Winogradsky and by him named *Cl. pasteurianum*. It is probably identical with, or very closely related to, an organism listed in Bergey's Manual as *Cl. butyricum*.

**Genus *Rhizobium*.**—Perhaps the most important group of

nitrogen-fixing bacteria is composed of certain species which, although surviving in the soil, are not always found freely growing there like the *Azotobacter*. Their most characteristic activity and form is seen when they grow in the tissues of certain plant roots, where they live very well.<sup>1b</sup> For this reason they have been given the generic name *Rhizobium*.

Six species of the genus *Rhizobium* are described, named chiefly for the type or species of leguminous plants with the roots of which they are characteristically associated. They are *R. leguminosarum*, associated especially with peas and vetches; *R. phaseoli*, infecting bean plants (genus *Phaseolus*); *R. trifoli* common in red, white, crimson and related clovers (genus *Trifolium*); *R. lupini*, especially



Fig. 192.—Cells of *Rhizobium meliloti* showing fat vacuoles and other portions of the cell. (Lewis, I. M., J. Bact., Vol. 35.)

effective in connection with the lupines (genus *Lupinus*); *R. japonicum* invading the soy bean (*Soja japonica*); and *R. meliloti* which is used in planting alfalfa and sweet clover (genus *Melilotus*)<sup>2, 3, 3a</sup>. All have in common the property of invading certain root hairs of different leguminous plants (beans, peas, clover, etc.). The invasion induces a small nodular reaction on the part of the plant, somewhat resembling a tubercle (Fig. 191). The bacteria are somewhat specific as to plant host. Thus, *Rhizobium japonicum* produces nodules on the soy bean, whereas *Rhizobium meliloti* will not do so, but the latter bacterium will produce nodules on species related to *Melilotus* such as (alfalfa) (*Medicago sativa*). In the nodules the bacteria live symbiotically with the plant. As will be seen, this is of very great benefit to agriculture, as the nodules aid in gathering nitrogen.



Strains of any given species vary greatly in respect to nodule formation, some being "good," others "poor." Good strains form relatively small numbers of large, pinkish nodules near the main roots. These root nodules, or pictures of them, have doubtless been seen by every student of elementary biology and they can be found on clover, peas, beans and other legumes in any garden.

The bacteria themselves are of peculiar form and are said to pass through various complicated life stages. However, it has been shown by Lewis that these "life stages" are merely periodic appearances due to the accumulation of reserve food granules, fat vacuoles and the like (Fig. 192). The bacteria may be observed in smears

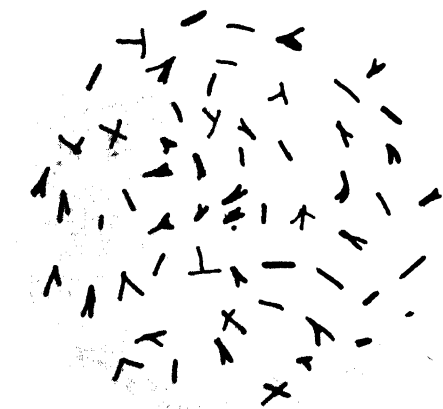


Fig. 193.—Various forms of *Rhizobium* ( $\times 900$ ).

made from crushed root nodules as Y-, L- and T-shaped, stellate or star-shaped, and ordinary rod-shaped cells (Fig. 193). They are motile when young. They are non-sporeforming, strict aerobes and may be cultivated on such materials as pea-broth gelatin containing asparagine, or such broth as the following:

H <sub>2</sub> O	1000.0 cc.
Glucose	20.0 gm.
KH <sub>2</sub> PO <sub>4</sub>	1.0 gm.
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5 gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1 gm.
NaCl	Trace
FeSO <sub>4</sub>	Trace
MnSO <sub>4</sub>	Trace
CaCl <sub>2</sub>	Trace

Agar may be added to stiffen the medium. Adjust the pH to about 7.2.

Note that in this medium a source of combined nitrogen is included since the bacteria are not growing symbiotically *in vitro*.

They utilize various carbohydrates, forming characteristic gummy or mucilaginous substances in cultures. These gummy coverings are of importance in helping them invade plant roots, forming a sort of hollow thread through which the cells migrate into the tissues<sup>3, 4</sup> (compare with the stalks of *Myxobacteriales*).

**Symbiotic Nitrogen Fixation.**—The rhizobia utilize carbohydrates and other substances found in the juices of the host plant and, like the *Azotobacter*, take their nitrogen directly from the air. This is synthesized by them to compounds from which it is yielded to the plant in which they live. The plants, when young, can utilize fixed nitrogen (nitrates) in the soil, but if this is absent and the proper species of *Rhizobium* for nodule formation are not present in the soil, they cease growth and die for lack of nitrogen. For this reason it is customary to inoculate virgin soils, or soils not known to support growth of legumes, with the proper species of *Rhizobium* preparatory to planting such crops as alfalfa or soy beans for the first time. State and Federal departments of agriculture often make suitable cultures available to farmers. They are also available on the market under various trade names. The cultures are usually mixed with the seed before planting.

The root nodules of legumes contain large amounts of combined nitrogen prepared by the bacteria and thus it is that the cultivation and plowing under of crops like clover, vetch or soy beans enriches the soil with "fixed" nitrogen as well as with the other valuable organic constituents of the whole plants. Inoculation of swampy acid soils is money wasted, as *Rhizobium* will survive and grow only in fertile, dry, aerated and neutral, or nearly neutral, soils. Crops of nonlegumes grown in association with legumes (as vetch and rye or clover and corn) have for centuries been known to be superior. The legumes apparently excrete fixed nitrogen into the soil.<sup>3, 4</sup>

**The Nitrogen Cycle.**—The nitrifying, nitrogen-fixing, and related bacteria described in the foregoing pages are of interest and value because they introduce into the soil in available form an element (nitrogen) which is absolutely essential to life and which is chemically so inert that it never combines spontaneously or readily with other elements, but only at the expenditure of great quantities of energy such as is found in lightning flashes or in huge electrical furnaces or other expensive apparatus. The bacteria produce combined nitrogen very cheaply, efficiently, and unostentatiously.

However, were all nitrogen continuously to be used by living

things and combined in the form of protoplasm and to remain so, inextricably bound up as protein, then the agricultural use of manures, animal carcasses, fish fertilizers, etc., would be of no avail. Were dead animals not to decay, manure not to rot, and dead fish to remain dead fish, the only form of nitrogen available would be free atmospheric nitrogen and all plants would have to await the slow activities of the nitrifying or nitrogen-fixing bacteria, or be limited to the use of the rare atmospheric ammonia or to ammonium salts, which are not very favorable, in order to obtain properly combined nitrogen. Such, however, is not the case.

*Organic Decomposition.*—As soon as protoplasm ceases to live, and as soon as any organic matter returns to the soil, it begins to undergo spontaneous oxidative changes and also the biological decomposition process of *decay*, which is aerobic decomposition, or *putrefaction* and *fermentation*, which are anaerobic decomposition of proteins and carbohydrates, respectively. Through these processes the nitrogen and other elements become available to plants. Decomposition results from the action of the hordes of bacteria and other creatures found in all soil and in natural waters.

The bacteria causing decomposition are, for the most part, saprophytes and heterotrophs but are frequently capable of autotrophic existence. There are numerous aerobic and anaerobic forms: cocci, bacilli and spirochetes. Each attacks one or more of the complex substances which compose dead plants and animals—cellulose, bone, hair, proteins, fats, carbohydrates—hydrolyzing or otherwise decomposing them and utilizing them as sources of energy or carbon or oxygen or nitrogen or some other element. One species of bacterium may utilize and decompose the metabolic products formed by another, and so on, until the complex organic matters become so simple, chemically, that they are again available as food for plants (Fig. 194).<sup>8</sup>

The bacteria of the soil thus live in a constant state of mutual assistance or *symbiosis*, interdependence, and competition and antagonism. So active are bacteria in attacking organic matter in the soil and utilizing the various elements of which it is composed, especially nitrogen, that the plowing under of manure or straw or other organic fertilizer just before planting may prove deleterious to the crop. The bacteria multiply in the fertilizer to such an extent as to offer serious temporary competition to the crop plants for the available nitrogen and other elements. Some bacterial products (acids, etc.) are also deleterious to crops and to other bacteria. Liming of the soil is then desirable. Later, of course, the bacteria die and disintegrate, liberating their nitrogen.

With regard to the nitrogen of the organic matter being decomposed, which alone concerns us for the moment, it is released from

protoplasm and from animal and plant wastes chiefly through decompositions involving *ammonification*, *deamination* and *denitrification*.

*Ammonification and Denitrification.*—In a previous chapter an outline was given of the biological processes by which protein is first hydrolyzed to amino acids and these broken down to other, simpler compounds when they are utilized as energy sources or in cell synthesis by bacteria and fungi. Ammonia resulting from deamination is commonly produced in bacterial protein decomposition.

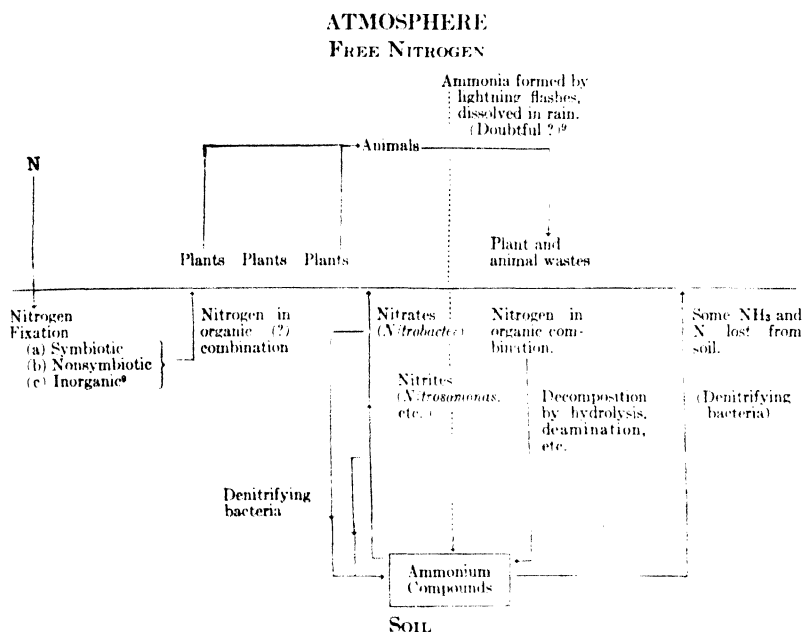


Fig. 194.—The nitrogen cycle.

The release of ammonia from the decomposition of organic nitrogen compounds is called *ammonification*. Note that the nitrogen is in its most reduced form (compare with  $\text{H}_2\text{S}$ ). Sulfur and nitrogen, as well as carbon and oxygen, are good hydrogen acceptors.

The fixed nitrogen represented by ammonia might escape, in part, from the soil, in the same manner that much is lost to the atmosphere from manure and compost heaps. It would then be lost to the living cycle were it not for its combination as ammonium salts and for the bacteria (*Nitrosomonas*) which oxidize a portion

of it to nitrites (nitrosification). The nitrites would be in great part useless to plants were it not for other bacteria (*Nitrobacter*) which oxidize them to nitrates (nitrification). Nitrosification and nitrification are often loosely spoken of together as nitrification.

The reverse of nitrification is referred to as *denitrification*. In denitrification, nitrates and nitrites are used by various bacteria of the soil to act as hydrogen acceptors, both nitrates and nitrites undergoing reduction. The extent of the reduction process depends on the species of bacteria involved and the availability of free oxygen. Many aerobes and anaerobes reduce nitrates to nitrites. This step in the process of denitrification is commonplace, nitrates apparently yielding one molecule of oxygen with little difficulty. Some familiar species of facultative bacteria reduce nitrites to nitrogen but this process is not so common a process as nitrate reduction. The nitrogen may even be further reduced to ammonia, and thus denitrification may proceed to ammonia formation. Some bacteria are incapable of anaerobic growth unless nitrates or similar compounds are present to furnish an acceptor of hydrogen.

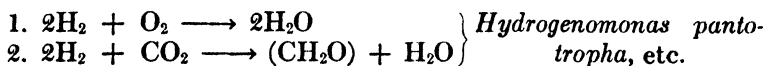
The reduction of proteins and of nitrates with liberation of nitrites, nitrogen or ammonia accounts in part for the lack of fertility of constantly wet soils containing organic and other matter furnishing food for ammonifying or anaerobic denitrifying species. Some of these species might be *Thiobacillus denitrificans* or various clostridia.

Thus we see that nitrogen, like sulfur, is in a constant state of alternation between an oxidized state and a reduced state as long as it remains in the living system. Its introduction from the inert atmospheric state into the cycle of life is almost entirely dependent on bacteria.

#### THE "PRIMITIVE BACTERIA": (TRIBE PROTOBACTERIACEAE)

Certain bacteria of the soil are of considerable interest not because of their role in the nitrogen cycle but because of their curious metabolism, especially their sources of energy. One group of these bacteria is called the genus *Hydrogenomonas*. These oxidize hydrogen as a source of energy and use part of this energy in cell synthesis.

The reactions are probably as follows:



These bacteria inhabit chiefly swampy places where organic de-

compositions are releasing considerable amounts of free hydrogen. They are facultative autotrophs, motile rods, and form heavy capsules around themselves. Some species are said to form spores. They may be isolated, after some experience, on organic media like-milk-agar or nutrient gelatin. Hydrogen, of course, is an efficient fuel, and its oxidation, resulting in the formation of water, yields abundant energy, part of which may be used in the chemosynthetic reduction of carbon dioxide as shown in reaction 2 above. But surely it is a strange and unsubstantial food; think of a delicious hydrogen stew!

Other primitive bacteria of the soil have similar curious metabolic properties and are included in the genera *Carboxydomonas* and *Methanomonas*. These oxidize simple carbon compounds as energy sources, as follows:

1.  $\text{CO} + \frac{1}{2}\text{O}_2 \longrightarrow \text{CO}_2$ . *Carboxydomonas oligocarbophila*
2.  $\text{CH}_4 + 2\text{O}_2 \longrightarrow \text{CO}_2 + 2\text{H}_2\text{O}$ . *Methanomonas methanica*

The organisms of these genera are very similar to each other and it is doubtful if they belong in different groups. Like *Hydrogenomonas*, they are small, motile rods growing in marshes and swamps where hydrogen, carbon monoxide and methane are bubbling up owing to the anaerobic decomposition of the organic matter, chiefly cellulose, in the depths of the bog. The *Carboxydomonas* are said to be strict autotrophs, while *Methanomonas* are facultative heterotrophs. They are aerobic organisms. Two interesting species of the genus *Methanomonas* are *M. aliphatica* and *M. aliphaticoliquefaciens* which oxidize the higher homologues of methane, such as certain of the petroleum oils and paraffin. Many other bacteria can attack and decompose hydrocarbons in gasoline, etc., and may be of considerable importance in the petroleum industry as causes of spoilage. Among these, as shown by Stone, Fenske and White and others are *Pseudomonas* and *Achromobacter* species, also *Alcaligenes*, etc.<sup>5, 6, 6a, 6b</sup>

The use of such bacteria, and of marine forms having similar properties,<sup>6a</sup> to find hidden sources of petroleum is of interest. Culture mixtures, complete in all respects except carbon source, are placed in flasks and inoculated with an appropriate species of organism able to utilize petroleum vapors as carbon source. On being lowered into suspected oil-bearing strata and left for some days, growth will occur if hydrocarbon (petroleum) vapors are present. Thus the bacteria in the hands of skillful bacteriologists serve as a sensitive reagent for petroleum wells.<sup>6b</sup>

**The Sulfur Bacteria (Genus *Thiobacillus*).**—We have already indicated that sulfur is one of the biologically essential elements which undergo cyclical alternations between living and nonliving systems. Here we shall discuss certain bacterial oxidative changes in sulfur analogous to the oxidative changes brought about by bacteria in nitrogen as a first step in the nitrogen cycle.

The bacteria involved may be divided into two large groups, often called the ectothiobacteria and the endothiobacteria. Most of the former are true bacteria (Eubacteriales) while the latter are grouped in a separate order, the Thiobacteriales, already described. Some ectothiobacteria (Athiorhodaceae) are also included with Thiobacteriales because they are photosynthetic (see page 394).

The ectothiobacteria (except Athiorhodaceae) are like other true bacteria except for the fact that they utilize inorganic sulfur compounds as sources of energy. They are grouped in the genus *Thiobacillus*. These organisms oxidize sulfur or its compounds in various ways, two of which are as follows:

1.  $5\text{Na}_2\text{S}_2\text{O}_3 + \text{H}_2\text{O} + 4\text{O}_2 \longrightarrow 5\text{Na}_2\text{SO}_4 + \text{H}_2\text{SO}_4 + 4\text{S}$   
 $4\text{S} + 6\text{O}_2 + 4\text{H}_2\text{O} \longrightarrow 4\text{H}_2\text{SO}_4$ . (*Thiobacillus thioparus*)
2.  $2\text{S} + 3\text{O}_2 + 2\text{H}_2\text{O} \longrightarrow 2\text{H}_2\text{SO}_4$ . (*Th. thiooxidans*)

Some species are said to thrive on sulfides or hydrogen sulfide. Thiobacilli are small, gram-negative, nonspore-forming, rod-shaped bacteria, some of which are motile, others nonmotile. They thrive in mud, sea water, and boggy places where sulfur and its compounds are being liberated as a result of protein decomposition, or where it exists free, as near sulfur springs. They differ from the endothiobacteria in *not* storing sulfur granules inside their cells as reserve food, a habit which is distinctive of many of the Thiobacteriales. On the contrary, the thiobacilli liberate free sulfur *outside* of the cell.

Among the most interesting and completely studied species of this genus are *Thiobacillus thioparus*, *Th. thiooxidans* and *Th. denitrificans*. These are strict autotrophs. Solutions like the following meet all of their nutritive requirements:

$\text{H}_2\text{O}$ .....	100.000 cc.
S.....	1.000 gm. or
$\text{Na}_2\text{S}_2\text{O}_3$ .....	0.500 gm.
$(\text{NH}_4)_2\text{SO}_4$ .....	0.030 gm.
$\text{KH}_2\text{PO}_4$ .....	0.025 gm.
$\text{CaCl}_2$ .....	0.050 gm.
$\text{FeSO}_4$ .....	0.001 gm.

*Thiobacillus thioparus* oxidizes sodium thiosulfate, hydrogen sulfide or sulfur aerobically. The last is transformed quantitatively

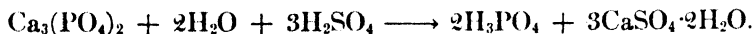
into sulfuric acid. Imagine giving off sulfuric acid as a waste product! One would become vitriolic without making any voluntary effort.

*Thiobacillus denitrificans* oxidizes sulfides and sulfur under strictly anaerobic conditions, obtaining its oxygen for this purpose from nitrates. This is of especial interest since respiration, or oxygen utilization, is often taken by the layman to mean the utilization of free atmospheric oxygen to oxidize organic matter with the liberation of carbon dioxide as a result of respiration. In the case of *Th. denitrificans*, the oxygen is obtained from nitrates, the material oxidized is sulfur or its inorganic compounds, and the product of respiration is sulfuric acid! It is also of interest because this represents one of the factors responsible for losses of fertility in certain soils—*denitrification*, or reduction of nitrates.

*Thiobacillus thiooxidans* oxidizes sulfur to sulfuric acid aerobically. As sulfuric acid is formed in considerable amounts, it might be thought that the organisms would quickly inhibit their own further growth. This species, however, is of interest in having a great resistance to acid. It is "distinctive in that it is able not only to tolerate but to produce higher concentrations of acid than any other living organism yet known" (Starkey). Some growth is said to occur at a *pH* of 1, and it grows readily at *pH*3, but it is readily killed by heat (50° C.) and is quite sensitive to drying.

Free sulfur is deposited by some species, as *Th. thioparus*, in granules *outside* the cell proper and forms a scum on flasks of medium. Other sulfur bacteria, as *Th. thiooxidans*, may oxidize it further to sulfuric acid.<sup>7</sup>

The importance of these sulfuric-acid-forming bacteria as agents in the acid-disintegration of various rocks and minerals, with the liberation of such valuable elements as phosphorus, magnesium, and sodium, must be borne in mind when considering the functions of bacteria in soil fertility. Thus, rock phosphates ( $\text{Ca}_3(\text{PO}_4)_2$ ) may be transformed to soluble phosphoric acid:



The transformation of barren alkali soils in desert places to fertile ones may be brought about through the activities of such organisms under conditions of irrigation.

*Functions of Sulfur Bacteria.*—The nitrogen of these curious creatures (genus *Thiobacillus*) may be obtained from ammonium phosphate or other inorganic nitrogenous salts not available to higher plants. Carbon dioxide is their source of carbon. They are of impor-



tance in making sulfur available for other living creatures and thus furthering the *sulfur cycle*. Sulfur, like nitrogen, is absolutely essential to the formation of protoplasm, and its addition to soil is an agricultural necessity. However, sulfur, like nitrogen, is available to higher plants chiefly in highly oxidized form ( $\text{SO}_4$ ), and the sulfur bacteria are vital sources of sulfates in the soil. A cyclical interchange of sulfur in various stages of oxidation between various living forms is thus seen, and constitutes the basis of a *sulfur cycle* which is, in many respects analogous to the nitrogen cycle (Fig. 195).

**The Sulfur Cycle.**—Hydrogen sulfide and various oxides of sulfur are given off into the atmosphere by volcanic action and are washed into the soil by rain. Sulfur springs also contain such compounds

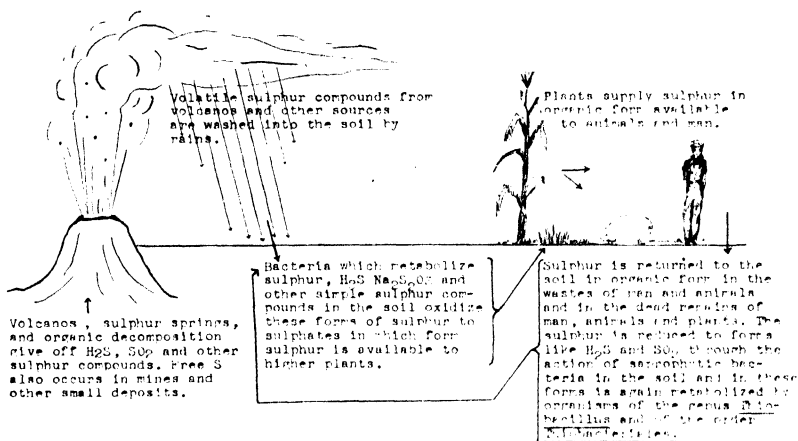


Fig. 195.—Diagram of the sulfur cycle.

as well as salts of sulfurous acid, while plant and animal proteins yield hydrogen sulfide upon decomposition. Pure, elemental sulfur is found in deposits in various parts of the world.

The higher plants, however, can utilize none of these, requiring sulfur in the form of sulfate. Animals, depending as they do on plants, therefore depend upon a source of sulfate. Bacteria are extremely important in supplying this necessary substance and the sulfur- and sulfide-metabolizing bacteria are therefore as important to higher life as the bacteria engaged in the production of nitrates and in denitrification.

The bacteria discussed under the heading *Thiobacillus*, as well as those included in the order *Thiobacteriales*, make use of elemental sulfur, hydrogen sulfide and thiosulfates, as sources of hydrogen

or energy, oxidizing these substances eventually to sulfuric acid which combines with carbonates, etc., to form sulfates. In this form sulfur is used by the higher plants and so the bacteria contribute to the support of the human race.

The higher plants and animals build the sulfates into their protoplasm. When they die they are decomposed and most of their sulfur is reduced by the bacteria and other fungi bringing about the decomposition (such species as *Proteus*, *Clostridium sporogenes*, *Cl. putrificum*) to free sulfur, hydrogen sulfide and other sulfur compounds like mercaptans, etc. In addition, there are several species of soil bacteria which reduce sulfates with the production of hydrogen sulfide. An example is the *Microspira desulfuricans* of Beijerinck. Several other spiral or curved sulfate-reducing organisms are known, which might be included in the genus *Desulfovibrio*. This power of reducing sulfates is not common in bacteria, and is analogous, with respect to soil fertility, to nitrate reduction. It is essentially anaerobic.

Some species of sulfate-reducing bacteria have been found causing damage to water systems. They reduce sulfates in the water to hydrogen sulfide which attacks the iron pipes, forming iron sulfide.

Sulfur in reduced forms is of especial usefulness to the sulfur-oxidizing bacteria in the soil and water, and so the sulfur passes back and forth from the living to the inorganic systems in an alternately reduced and oxidized form. Its initial introduction into the living scheme from its mineral or atmospheric status is largely by means of the sulfur-oxidizing bacteria, while its return to these states is by means of sulfur-reducing species.

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## CHAPTER 26

### BACTERIA COMMONLY FOUND IN WATER

**Sources of Water and Bacteria Therein.**—Water may be considered in two general categories, fresh water and sea water. Fresh water may be *ground water*, as subterranean streams; or *surface water*, as lakes, springs or rivers. Ground waters such as those of deep springs and artesian wells are usually sterile, because the bacteria in the surface waters from which they are derived are filtered out by the soil. However, as soon as they become surface waters, they acquire a floating population from the surface soil, banks of streams, and other sources.

*Fresh surface water* cannot be said to have any characteristic bacterial flora, the kinds of bacteria in it depending on its mineral and organic content, the soil with which it is in contact, surface pollution and numerous other factors. Rainfall increases the numbers of soil bacteria in the waters into which it drains. The lower Hudson River has a flora representative of heavy sewage pollution; *Escherichia coli* and other intestinal bacteria such as *Streptococcus faecalis* and *Clostridium perfringens* are present in large numbers, as well as the soil saprophytes like *Pseudomonas*, *Proteus* and *Sarcina* which find the organic fecal matter good pabulum. The nature and extent of the bacterial populations in streams where cattle wade will obviously differ from those in high mountain torrents derived from melting snow. The Chlamydobacteriales and Thiobacteriales

are common in iron-bearing and sulfur-bearing waters, respectively, sewage being a sulfur-bearing water. In most unpolluted lakes and streams one may find various members of the Caulobacteriales. Common in most surface waters are various species of *Micrococcus* and of *Serratia*, *Chromobacter*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, and *Pseudomonas*, as well as of *Proteus*, *Bacillus*, *Aerobacter*, *Lactobacillus*, *Clostridium*, *Vibrio* and various actinomyces, yeasts and molds. Many other bacteria common to the soil also occur in most surface waters.<sup>1</sup> The Great Salt Lake of Utah has its own peculiar flora of halophilic bacteria capable of living in strong salt solution.<sup>2</sup> These have been discussed previously.

*The open sea*, as shown by ZoBell, Stanier and others may be said to have its own indigenous flora, but even here we find differences in surface flora and the populations of the profound depths. *Thiobacteriales* are common in the sea. One of the many interesting groups of marine species includes photogenic (light-producing) bacteria.

*Photogenic Bacteria*.—There are several species of photogenic bacteria, many of which occur in the sea and which may be cultivated upon sea-water agar. Some require peptone. *Pseudomonas phosphorescens*, isolated from luminous marine fish, is one example of a saprophytic species. At times, photogenic bacteria may grow thickly on the surface of fish or other water animals, causing luminescence (Fig. 196).<sup>3</sup> In olden times, certain persons are said to have been regarded as holy or as having miraculous powers because they gave off light from their bodies. An explanation of these phenomena, offered in view of our present knowledge of luminescent bacteria is that, under vows never to bathe, the skin of certain persons became a suitable nutrient surface for photogenic bacteria.

Marine bacteria have received all too little attention. Many of them may be ancestral varieties of terrestrial forms. A number of species of agar-digesting and chitin-digesting bacteria as well as other interesting types have been described in sea water by ZoBell, Stanier, Henrici, van Niel and others. Similar forms occur in the soil. Some resemble members of the soil genera *Flavobacterium*, *Pseudomonas* and *Cellulomonas*; others are like *Cytophaga* and *Vibrio*. They are adapted to a marine life and adversely affected by solutions of low salinity. Some are autotrophic.<sup>4, 5, 6, 7</sup> An interesting nitrogen cycle is carried on by marine bacteria.<sup>7a</sup> Some marine species attack petroleum hydrocarbons (see page 417).

Among the bacteria not wholly indigenous to the open sea but found very commonly in fresh and brackish waters, especially if

sewage-polluted, are members of the genera *Pseudomonas*, *Proteus*, and *Serratia*. These also occur in many other situations such as soil, dung, etc. They are related and are similar in many respects. All are gram-negative rods; about  $2\ \mu$  by  $10\ \mu$  in dimensions although varying considerably in size and form at times; non-sporeforming; mostly facultative although some are strict aerobes; growing readily on simple media; mostly motile; fairly active in fermentation of a variety of carbohydrates and in organic decomposition in general, differing from each other in specific details; and regularly inhabiting

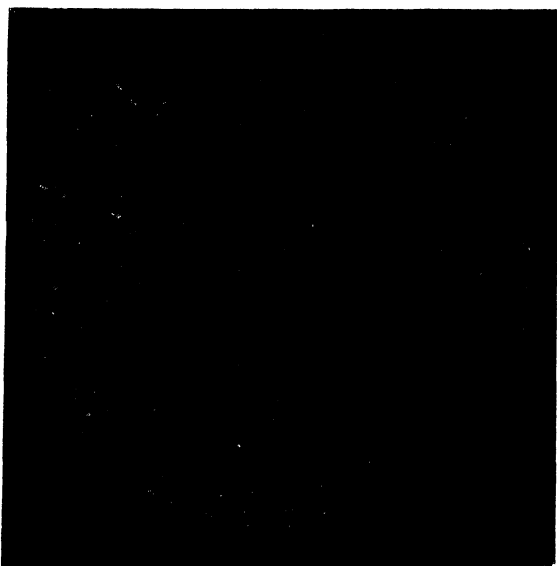


Fig. 196.—Colonies of photogenic bacteria. (Lafar.)

the soil and ground waters. Because they are of considerable importance in sanitary bacteriology, industrial spoilage, experimental procedures and other relationships which make a knowledge of them very desirable, they will be discussed here in some detail.

**Genus *Pseudomonas*.**—These organisms are widely distributed in nature. They are motile and actively proteolytic. *Pseudomonas fluorescens* is present in all fertile soils and surface waters. It produces a greenish-yellow fluorescent pigment in cultures containing organic nitrogen (peptone).<sup>8</sup> Several other species of *Pseudomonas* are well-studied organisms and serve to illustrate important bacteriological phenomena. Thus, *Pseudomonas aeruginosa*, type species

of the genus, is of special interest. It is distinguished from other members of the genus by the production, in addition to the yellow-green pigment, of a turquoise-blue pigment, *pyocyanin*, which may be extracted from broth culture with chloroform. This organism is not infrequently found in wounds or ulcers which have not healed promptly. It causes the pus in such situations to turn a greenish-blue color, hence its older name (*pyo* = pus; *cyaneus* = blue). It probably has not the power of invading healthy tissues but sometimes gains a foothold and grows in already diseased foci, living a saprophytic existence there, possibly producing substances which result in the death and destruction of already injured tissue cells. It takes advantage of such opportunities for infection and is another example of a bacterial opportunist. Elrod and Braun have shown *Ps. aeruginosa* to be able to cause a leaf-rot disease in tobacco and lettuce.<sup>9</sup> *Ps. aeruginosa* also causes a fatal disease in poultry.

**Blue Milk.**—The organism most commonly responsible for this curious condition is closely related to *Pseudomonas aeruginosa* and is called *Ps. synchyanea*.<sup>10</sup> It resembles *Ps. aeruginosa* in many respects and gains entrance to the milk from soil.

The growth of this bacterium in milk which has become slightly acid (due to the simultaneous growth of *Streptococcus lactis*) results in a deep blue color. The pyocyanin-like pigment acts in the manner of an acid indicator, for it is colorless or brownish in fresh or alkaline milk.

In considering the species of organisms of the group of gram-negative, non-sporeforming rods of the soil, water and intestine represented by the germs *Pseudomonas* one need not regard each of them as an isolated, well-differentiated, sharply defined subdivision, but as part of a sort of spectrum of bacterial species, very subtly blended, some species of which are distinctly different from others and therefore constitute type species of genera, but which grade by almost imperceptible differences and combinations of characters into other species or genera which have somewhat different characters; yet they all have certain properties in common. From this viewpoint one sees many relationships that otherwise would not be appreciated. An example is the similarity of one group of *Phytomonas* species to the *Pseudomonas*. The resemblance is striking and includes the production by both groups of a water-soluble greenish-yellow pigment (see section on plant diseases, page 464).

**Genus *Proteus*.**—A second relationship is seen in the likeness between *Pseudomonas* and *Proteus* (Fig. 197). The latter comprises

a group of soil and water saprophytes, common in decaying animal or vegetable matter and often found in the human intestine or as opportunists in infections (especially cystitis) of man and causing diseases in lower animals.<sup>11, 13</sup> These are also situations in which species of *Pseudomonas* are sometimes found.

The *Proteus* species, while grouped with the Enterobacteriaceae because of their ability to grow in the intestinal tract and their fermentative powers, resemble the *Pseudomonas* in many respects, but do not produce pigment. *Proteus* might be regarded as *Pseudomonas* which have lost their power of pigment formation. However, *Pseudomonas* have polar flagella, while *Proteus* have peritrichous

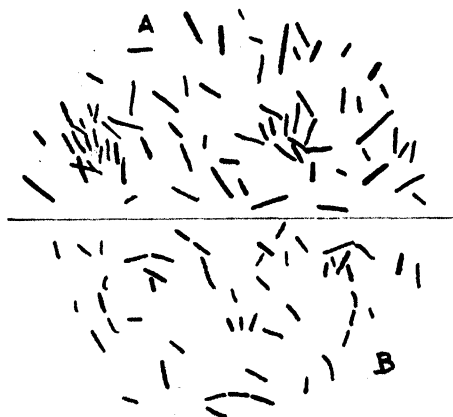


Fig. 197.—Similarity of members of different families. A *Pseudomonas fluorescens* (family Pseudomonadaceae); B *Proteus vulgaris* (family Enterobacteriaceae) ( $\times 900$ ).

flagella. Further, *Proteus* colonies are ordinarily distinctive in that they spread rapidly over the surface of solid media (see “swarming,” page 253), forming a thin, gray, almost transparent film which may escape notice entirely unless it is especially sought. In the (O) phase of variation, however, the colonies are discrete and circular. As will be seen later, certain varieties of *Proteus* have a peculiar interest in connection with the diagnosis of Rocky Mountain spotted fever and related diseases, being agglutinated by the serum of patients with these infections.<sup>12</sup>

*Proteus hydrophilus* is of interest as the cause of a natural disease, “red leg,” of frogs. The frogs seem to harbor the microorganisms in

the gallbladder. The colonies of *P. hydrophilus* are of the O type but are motile.<sup>13</sup>

*Serratia marcescens*, type species of the genus *Serratia*, may be specially mentioned, although the interest attached to it was originally more philosophical than scientific.<sup>14</sup> This bacillus multiplies rapidly, producing a blood-red pigment, especially when growing upon starchy foods at room temperatures. It is a common facultative, motile saprophyte of the soil and water and is found on bread and in milk and other foods. Due to its rapid growth and red coloration and digestion and liquefaction of the substrate in the presence of adequate moisture, it often produces a condition of bread called "bloody bread." It has been shown that color changes observed in



Fig. 198.—*Serratia marcescens* ( $\times 900$ )

sacramental wafers are due to the growth of *Serratia marcescens* and the bacterium has consequently been referred to as the "miracle organism." Capable of such prodigious things, it was given the name of *Bacillus prodigiosus* by one author. However, it was later named *Serratia marcescens* for a famous Italian physicist, Serrati. The name *marcescens* indicates its hydrolytic activity. It is of industrial importance as a cause of spoilage in improperly stored milk, producing its red pigment and "red milk."

In size, it is one of the smallest bacteria and for this reason is frequently used as a control organism in testing the efficacy or fineness of bacterial filters. Due to its rapid growth and distinctive color, it is readily detected in filtrates from defective filters (Fig. 198).



*Serratia marcescens* is of further interest as one member of an hypothetical "metabolic gradient" which, for purposes of discussion, may be postulated to exist in the group of pathogenic intestinal bacteria to be discussed in Chapter 29 (tribe Salmonelleae).

This hypothetical gradient (admittedly a very rough one) has at its *upper*, pathogenically active but metabolically inactive, extreme the genus *Shigella*, the cause of dysentery, and the habitat of which is confined largely to the human intestine. The shigellas cannot live for extended periods in the soil, since they are accustomed to life in a parasitized host.

At the *lower* end of the hypothetical gradient we find the metabolically active genus *Aerobacter*, a group of species which are primarily soil organisms, only occasionally being found in the intestine, and lacking in pathogenicity.

The genus *Serratia* might be regarded as an extension of the same gradient below *Aerogenes*, into the group of definitely and almost exclusively harmless soil and water types seldom or never found in the intestine—a sort of *infra-Aerogenes* group, so to speak, containing organisms like *Serratia marcescens*, even more active, metabolically, and more resistant to "outdoor life" than *Aerogenes*, and never found in the intestine. A signal exception is seen in the occurrence of *S. marcescens* in the spinal fluid of a patient with meningitis, seemingly in etiological relationship.<sup>11a</sup> *S. marcescens* has many properties of the *Aerobacter*, fermenting many carbohydrates actively, being motile, liquefying gelatin, coagulating and sometimes digesting milk, producing acetyl-methyl-carbinol, carbon dioxide, hydrogen, and a variety of organic acids and alcohols from dextrose, reducing nitrates, growing well at temperatures below that of the body (25° to 30° C.) and surviving well in the soil and water. This is in marked contrast with the organisms like *Shigella dysenteriae* found at the other end of the gradient, which are much more limited in the number of substances they can metabolize, and cannot grow in the outer world and soon die off there, are sensitive to drying and sunlight, and require specially prepared substances for their growth. Between these two extremes there are *Eberthella typhosa*, *Esch. coli*, etc., which represent gradations in metabolic activity and dependence upon a host of some sort for support (parasitism). (See page 267.)

**Genus *Aerobacter*.**—The organisms of this genus possess the general characters of the genera discussed above—mesophilic, gram-negative, non-sporeforming rods of moderate size (0.7 by 5  $\mu$ ). All are facultatively anaerobic, grow in relatively simple media and

live generally in the soil and in polluted water. They occur at times in the intestines of man or lower animals. The organisms are therefore included in the rather loose term "the gram-negative intestinal" or "colon-typhoid" group. Included in this loose and ill-defined intestinal group also are the *Escherichia* organisms and the genera of dangerous pathogens, *Eberthella* (typhoid), *Salmonella* (paratyphoid), and *Shigella* (dysentery) organisms, as well as *Proteus*.

The organisms of the genus *Aerobacter* are a sort of transitional form between gram-negative, non-sporeforming facultative rods like *Proteus* and *Serratia* which live only in the soil, and those which

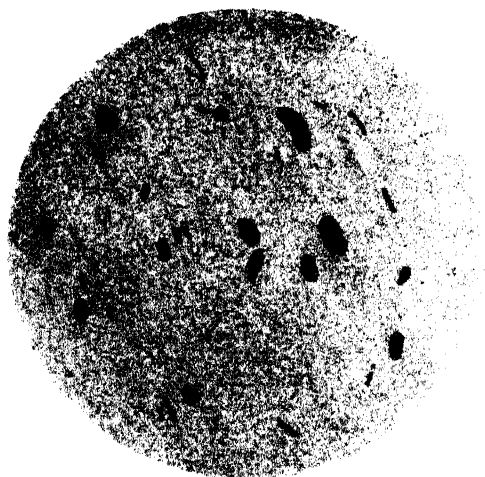


Fig. 199.—*Aerobacter aerogenes* ( $\times 900$ ).

have become adapted to life as a commensal in the animal intestine like *Escherichia coli*. The *Aerobacter* live in both environments, but perhaps are more commonly soil organisms than intestinal.

*Aerobacter cloacae* is most closely related of the *Aerobacter* to the true soil and water species. It is motile, liquefies gelatin and other proteins, is highly fermentative, produces acetyl-methyl-carbinol but does not produce pigment. It is predominantly a soil and water species like *Ps. fluorescens* or *Proteus vulgaris* but, like the latter, is occasionally found in the intestinal canal. An interesting species, either identical with or very similar to *A. cloacae*, was described by Schneider, Neal and Caminita<sup>14b</sup> in connection with a febrile, acute, cold-like disease of persons working in cotton mills. The or-

ganism is present in large numbers in the cotton fibers but does not appear to be a pathogenic parasite of the plant. The authors suggest that this species produces some substance which is poisonous to human beings.

*Aerobacter aerogenes* differs from *A. cloacae* in being frequently nonmotile, usually encapsulated (it produces motile, nonencapsulated variants) (Fig. 199) and in failing to liquefy gelatin. *A. aerogenes* is predominantly an environmental (or soil and water) organism, but is found in feces more frequently than *A. cloacae*.<sup>14c</sup>

**Genus *Escherichia*.**—Next removed from the distinctly saprophytic and free-living forms, we find the genus *Escherichia*, the most important species of which is *Escherichia coli* (Figs. 200 and 201). This is preeminently an in-



Fig. 200.—*Escherichia coli* ( $\times 900$ ).

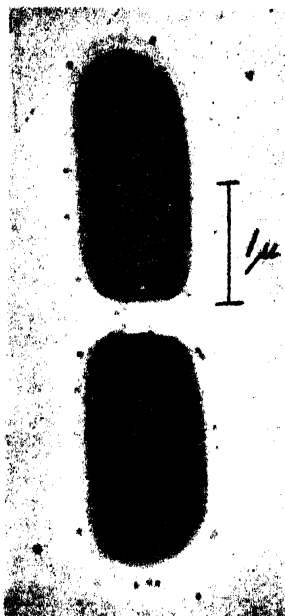


Fig. 201.—Electron microscope photograph of *E. coli* ( $\times 16,500$ ). The dark granules around the periphery of the cells are particles of bacteriophage (see page 745). Compare with Figure 200. (Luria, Delbrück and Anderson, Jour. of Bact., Vol. 46.)

testinal organism. *Esch. coli* is a little less active metabolically than *Aerobacter aerogenes* and is occasionally pathogenic. It does not form acetyl-methyl-carbinol, is not so invariably or actively motile as *A. cloacae* and fails to metabolize many of the substances which can be metabolized by *A. aerogenes* and *A. cloacae*.

*Escherichia freundii*, a species common in the soil and water, but

also frequently found in the normal human intestine,<sup>14c</sup> occupies a position between *Esch. coli* and the *Aerobacter*. It has important distinctive characters of both genera and has therefore been designated as an "intermediate species." On the other hand, because it resembles *Esch. coli* in some respects but resembles the *Aerobacter* in being able to utilize *citrates* as a sole source of energy or carbon in an otherwise wholly inorganic medium, it (and its variants) have been widely referred to as species under the generic name *Citrobacter*.

These four species (two of *Escherichia* and two of *Aerobacter*), because of their occurrence in polluted water, have a very important bearing on the health of everyone, a fact which needs some detailed discussion.

#### SANITARY RELATIONSHIPS OF THE GENERA *ESCHERICHIA* AND *AEROBACTER*

Every well informed person knows that *Eberthella typhosa* (the typhoid bacillus) is an intestinal parasite and that typhoid fever is transmitted by food, milk or water supplies which have become polluted with the urine or feces of a person harboring this organism. In thickly populated districts practically every body of surface water receives some fecal pollution due to the careless habits of ignorant people with respect to urination and defecation. Irrigation ditches and open canals are good examples. Sometimes people may be perfectly healthy yet harbor typhoid bacilli in their intestinal or urinary tract and discharge them in large numbers, thus infecting water supplies, milk, food and other substances which they handle. Such persons are called "carriers" of typhoid. Carriers of many other pathogenic bacteria are also common.

Now, it might seem a simple matter to detect typhoid bacilli in water supplies, since they are easily cultivated on nutrient broth or agar, but actually this is extremely difficult for many reasons and, until recently, had rarely been accomplished. The bacilli do not seem to live for more than a few weeks, usually not more than a month or so at most, in natural water due, in part, to the antagonistic action of free-living saprophytes like *Pseudomonas aeruginosa* and plankton organisms. Further, the numbers of typhoid bacilli in a lake or stream are usually relatively so small that, although enough might be present in a pint of water to cause disease, the practical difficulty of isolating *Eberthella typhosa* in pure cultures from such large amounts of fluid is very great.

However, it has been found that typhoid bacilli may be cultivated in such water by adding to it the ingredients of a peptone

medium in appropriate proportions, adding also a very small amount of *sodium selenite*\* which prevents the growth of antagonistic bacteria such as *Pseudomonas*.<sup>15</sup> Such a process is often called 'selective cultivation.'

Pollution of water with fecal material, whether infected or not, is obviously undesirable, both from the standpoint of its possible danger as a source of infection and for purely esthetic reasons. The detection in water of fecal bacteria of any kind is therefore of importance in determining its suitability for drinking purposes. Two very common, readily cultivated and numerically predominant types of intestinal organism are the intestinal streptococci and *Escherichia coli*. For every typhoid bacillus there may be millions of streptococci or *Esch. coli*. Streptococci are sometimes sought in sanitary examinations of water, especially in England, but in the United States *E. coli* is considered a more sensitive indicator of pollution and is more easily demonstrated.

In order to determine its presence, advantage is taken of the ability of *Esch. coli* to ferment lactose with the production of gas. But the *Aerobacter* and also *Esch. freundii* ferment lactose with gas production and it is therefore of importance to distinguish between the three because *Esch. coli* is definitely an intestinal organism while *Aerobacter* and *Esch. freundii* are also frequently derived from soil.

**Sanitary Bacteriological Examination of Water.**—The American Public Health Association has outlined an *official* procedure<sup>16</sup> for the isolation of *Escherichia* and *Aerobacter* from water and has described an *unofficial but useful* procedure for the differentiation between *Esch. coli* of truly intestinal origin and closely related, but less frequently intestinal, organisms like *Esch. freundii* and the *Aerobacter*. The value of the latter differentiation for official purposes is still in debate. Any fecal organism (*Escherichia* or *Aerobacter*) is officially (and rightly) regarded with suspicion. The details of these processes are given here in outline form only (Chart I).

\* Selenite medium for isolating typhoid bacilli from feces, sewage, or polluted water (Leifson):

For feces:

Sod. hydrogen selenite (anhyd.)	0.4 gm.
Na <sub>2</sub> HPO <sub>4</sub>	0.75 gm.
NaH <sub>2</sub> PO <sub>4</sub>	0.25 gm.
Peptone	0.5 gm.
Lactose	0.4 gm.
H <sub>2</sub> O	100.0 cc.

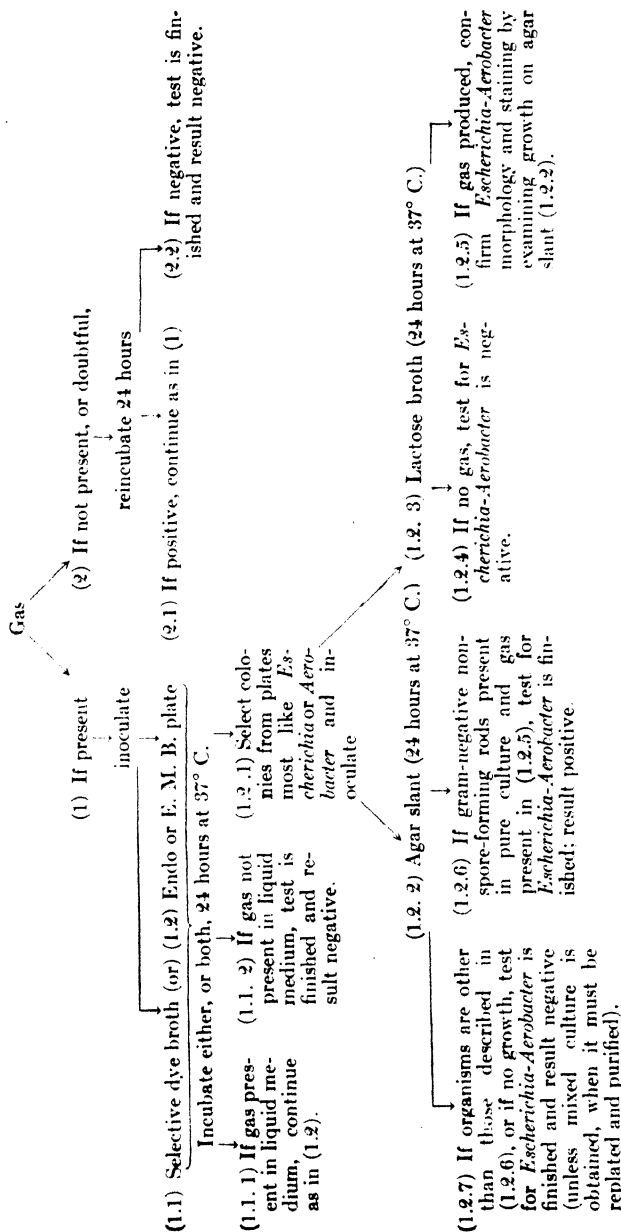
(Do not autoclave—heat at 100° C. for 30 minutes only.)

For sewage, increase the selenite to 1.5 percent and prepare the medium 10 times the indicated concentrations. Then dilute it with 9 volumes of the sewage or water.

CHART I

OUTLINE OF OFFICIAL TEST FOR *ESCHERICHIA-AEROBACTER* GROUPS\*

0.5 percent lactose broth fermentation tubes (24 hours at 37° C.)



\* Adapted from "Standard Methods of Water Analysis," 8th Edition, 1936, American Public Health Association, New York City, N. Y.

Briefly, the water to be examined is inoculated in measured amounts into tubes of extract—0.5 percent lactose broth, and is incubated for twenty-four hours. If gas is formed it is regarded as *presumptive* evidence that *Esch. coli* is present.

However, the gas may be due to *Aerobacter* (Sp.) or some other organism such as *Clostridium perfringens*, yeasts, or synergistic combinations capable of producing gas from lactose. The broth culture is, therefore, either streaked on an agar medium containing some dye which inhibits all but the *Escherichia* and *Aerobacter*, *e.g.*, Endo\* or eosin-methylene-blue† (see bacteriostasis, page 126), or transfers are made directly from the lactose broth to liquid media of similar composition (or based on the same principle) but containing no agar, *e.g.*, brilliant-green-lactose-bile broth.‡

**\* Endo agar:**

Distilled water	1000 cc.
Meat extract	5 gm.
Peptone	10 gm.
NaCl	5 gm.
Agar	30 gm.

Autoclave to melt all ingredients. Adjust to pH 7.4. Autoclave to sterilize.

Before using add to each 100 cc.:

- (1) Lactose, sterile 20 percent solution, 5 cc. (or 1 gm. dry powder).
- (2) Sodium sulfite ( $\text{Na}_2\text{SO}_3$ ), fresh 12.5 percent solution, 1 cc. (or 0.125 gm. dry powder).
- (3) Basic fuchsin (1 percent alcohol solution), 1 cc.

Pour plates and let them dry before streaking. Avoid exposure to light. One hundred cc. of the agar should make 6 to 8 plates.

**† Eosin-methylene-blue agar (Levine modification):**

Distilled water	1000 cc.
Peptone (Difco)	10 gm.
$\text{K}_2\text{HPO}_4$	2 gm.
Agar	30 gm.

Autoclave. No adjustment of reaction or filtration is necessary.

Before using, add to each 100 cc. of medium:

Sterile lactose solution (20 percent)	5 cc. (or 1 gm. dry powder)
2 percent aqueous eosin	2 cc.
0.5 percent aqueous methylene blue	2 cc.

Mix well and pour 6 to 8 plates.

**‡ Brilliant-green-lactose-bile broth (one form of liquid dye medium for detection of *Escherichia*-*Aerobacter* group):**

Peptone	10.0 gm.
Lactose	10.0 gm.
Ox bile	200.0 cc. (or 20 gm. of dehydrated bile dissolved in 200 cc. of water; pH 7.0 to 7.4)

Brilliant green (0.1 percent aq. sol.) . . . 13.3 cc.

Distilled water . . . . . 1000.0 cc.

If, after incubation of these liquid cultures, gas is produced, it is strongly suggestive of the presence of *Aerobacter* or *Escherichia* because most other species are inhibited by the dyes. The liquid dye cultures may, for further proof, be streaked on the dye-containing plates as mentioned above.

After the dye plates are inoculated (either directly from the original lactose broth or from the liquid dye media) they are incubated for 24 hours and colonies thought to be those of *Escherichia* or *Aerobacter* are fished, in pure culture, to lactose broth for verification of the purity of the culture and its lactose-fermenting properties. An agar slant is also inoculated for examination of staining and of morphology, proof of *aerobic growth* and for storage. If a gram-negative, non-sporeforming rod is found, it could hardly be any organism but the *Escherichia-Aerobacter* group, since no others would grow under the conditions provided.

The selection of *Escherichia* and *Aerobacter* colonies on these plates is facilitated by three factors: first, the plates contain lactose, readily fermented and acidified by these organisms; second, the plates contain dyes such as fuchsin, eosin and methylene blue, which inhibit many other bacteria; third, the acidification of the lactose under the *Escherichia* and *Aerobacter* colonies turns the dyes in the agar a distinctive color in a zone around each colony so that it is easily recognized.

This completes the official procedure and is designated as a "completed test." However, at this point we do not know more than that we have on hand organisms belonging either to the genus *Escherichia* or *Aerobacter*—the so-called "coli-aerogenes" group. For reasons already cited, additional tests are frequently made, to distinguish the *Aerobacter* from the *Escherichia*, and *Esch. coli* from *Esch. freundii*.

Formerly these differential tests were made with the pure cultures on the agar slants of the completed test referred to above, but experience has shown that, when one desires to determine the presence and numbers of *Esch. coli*, separately from *Esch. freundii* or the *Aerobacter*, it is preferable to inoculate melted and cool (45° C.) "selective" or "dye" agar with measured amounts of the water directly in selective dye plates instead of lactose broth. Desoxycholate agar is generally used (see section on typhoid, page 487). After incu-

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Dissolve lactose and peptone in 500 cc. water. Add bile, and make up with distilled water to 975 cc. Adjust to pH 7.4. Add brilliant green and make up to 1 liter; filter through cotton. Tube in 10 cc. portions (for 10 cc. samples of water). Sterilize in autoclave at 120° C. (15 pounds) for 15 minutes. Cool rapidly.



bation, the colonies of the distinctively colored *Escherichia* and *Aerobacter* groups may be counted and fished separately for further pure culture study. The cultures thus isolated are more apt to be pure than if isolated from lactose broth.

*Short Method for Escherichia-Aerobacter Group.*—A simpler and quicker procedure for demonstrating the probable presence of the *Escherichia-Aerobacter* group in water, milk, etc., consists in direct inoculation of the sample into tubes containing medium which is highly selective for these organisms. The lactose bile-brilliant green medium already mentioned is good for this purpose. The use of buffered lactose broth containing lauryl sulfate\* in 1:10,000 concentration as an inhibiting agent for extraneous organisms has given excellent results in the hands of Mallmann and Darby<sup>16</sup> and others, while Perry and Hajna<sup>17</sup> have obtained equivalent results with 0.15 percent bile salts as inhibitor in a medium ("E-C" medium) of similar composition.† Almost the only organisms known to be capable of producing gas in such media are those of the *Escherichia-Aerobacter* group.

The latter authors have shown that by making the original inoculations into tubes of buffered lactose broth containing no inhibitory agent *but incubated at 45.5° C.*, or by making secondary transfers from original "E-C" cultures showing gas into such broth, gas-formation is obtained only in the presence of *E. coli* which, alone of the group, has the power to produce gas *at this temperature*.<sup>18</sup>

These procedures are excellent illustrations of the practical application of selective bacteriostatic methods, and of an incubation temperature which is critical for the activity of an enzyme (formic

\* Lauryl sulfate tryptose broth (*Mallmann and Darby*)

Bacto-tryptose .....	20.0 gm.
Lactose .....	5.0 gm.
KH <sub>2</sub> PO <sub>4</sub> .....	2.75 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	2.75 gm.
NaCl .....	5.0 gm.
Sodium lauryl sulfate .....	0.1 gm.
Water .....	1000.0 cc.

Final pH 6.8. Tube and autoclave.

† "E-C" Medium (*Hajna and Perry*)

Bacto-tryptose .....	20.0 gm.
Lactose .....	5.0 gm.
KH <sub>2</sub> PO <sub>4</sub> .....	1.5 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	4.0 gm.
Bacto bile salts No. 3 .....	1.5 gm.
Na Cl .....	5.0 gm.
Water .....	1000.0 cc.

Final pH 6.9. Tube and autoclave.

decarboxylase) responsible for gas production by *E. coli*. From the standpoint of bacteriologists interested in legal aspects of the sanitation of water, objections to these short-cut procedures are (a) that the selective media may be too inhibitory and thus eliminate some strains of the *Escherichia-Aerobacter* group, and (b) that while the gas produced in the culture is *probably* due to these organisms, it is not *proof* of their presence since it is conceivable that some other species might act similarly under the conditions of growth provided. It is claimed that only the isolation and further study *in pure culture* called for by "Standard Methods" can eliminate this possibility. Only long experience with these new methods can determine the validity, or otherwise, of these objections.

**Differentiation of Fecal and Nonfecal Types.**—Having obtained satisfactory pure cultures, one makes differentiations be-

TABLE III  
DIFFERENTIAL FINDINGS, FECAL AND NONFECAL TYPES

	Indol	Methyl Red Test	Voges-Proskauer Reaction	Citrate Utilization	Gas Ratio (CO <sub>2</sub> /H)
<i>Escherichia coli</i> ...	+	+	—	—	1 : 1 or 1 : > 1
<i>Escherichia freundii</i> (Intermediates or Citrobacter).....	—	+	—	+	1 : 1 or 1 : > 1
<i>Aerobacter</i> .....	—	—	+	+	2 : 1 or 2 : < 1

tween *Esch. coli*, *Esch. freundii* and *Aerobacter* on the basis of indol production, the methyl red test, the Voges-Proskauer reaction and ability to utilize sodium citrate as a sole source of carbon (Table III). Another determination frequently made is that of the gas ratio. The test for indol has already been described. The technic of the other tests is described below.

**Imvic Formula.**—A mnemonic "*IMViC*" is used, with plus and minus signs, to express a sort of formula of these differences. *I* stands for indol reaction, *M* for methyl red reaction, *V* for the acetyl-methyl-carbinol test (originated by Voges-Proskauer), *i* is for convenience, and *C* for growth in citrate broth. Thus, an organism of the *Escherichia-Aerobacter* group designated as *IMViC* ++ -- would indicate *E. coli* since this gives positive indol and

methyl red reactions but negative Voges-Proskauer and citrate reactions.

**Citrate Utilization.**—The utilization of this ester in an inorganic medium, along with the properties mentioned above, distinguishes *Esch. freundii* from *Esch. coli*, which it otherwise very closely resembles. The test is simple, being merely an observation as to whether or not growth (as evidenced by turbidity) occurs in the simple solution already described (see page 158). In order to avoid

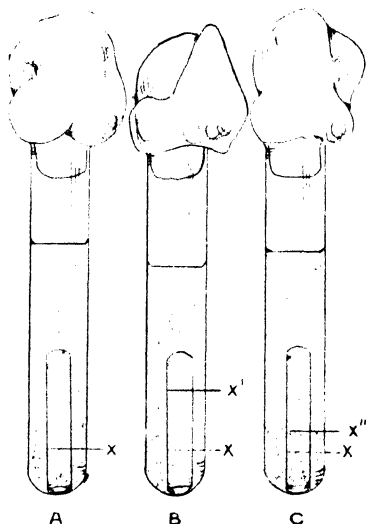


Fig. 202.—Diagram showing determination of gas ratio in Durham tubes. A, Total volume of gas mixture, X indicating height of fluid in inner tube before adding NaOH; B, after adding NaOH. Fluid has risen from X to X', showing the volume of CO<sub>2</sub> absorbed by the NaOH. The ratio of CO<sub>2</sub> to unabsorbed gas (H) is approximately  $\frac{\text{CO}_2}{\text{H}} = \frac{2}{1}$  (*Aerobacter*); C, after adding NaOH to a tube in which the gas ratio is approximately  $\frac{\text{CO}_2}{\text{H}} = \frac{1}{2}$  or  $\frac{1}{2}$  (*Escherichia*).

an initial turbidity, or the introduction of peptone, the inoculation must be light.

**Gas Ratio.**—The metabolic products evolved from dextrose by *Escherichia* and *Aerobacter* differ markedly. When *Escherichia* ferment dextrose they form acids and the two gases, hydrogen and carbon dioxide. The amount of carbon dioxide never exceeds the volume of hydrogen, the latter usually being present in excess. The *Aerobacter* produce the same gases but in an inverse ratio, i.e., carbon

dioxide always predominates in the mixture. If fermentation be allowed to occur in a closed tube so as to collect the gas, the amount of carbon dioxide may be estimated by mixing an equal volume of 10 percent solution of sodium hydroxide with the culture. This absorbs the carbon dioxide, leaving only the hydrogen, which may be measured by reference to a previously made mark (Fig. 202).

**Acetyl-methyl-carbinol** (*The Voges-Proskauer Reaction*).—When dextrose is fermented by the *Aerobacter* and some other organisms, such as *Serratia marcescens*, not only are acids and gases formed, but also a substance called *acetyl-methyl-carbinol*. (See Chapter 20.) This may be detected by adding strong sodium hydroxide to the culture and aerating well. The acetyl-methyl-carbinol undergoes oxidative changes to diacetyl and forms a compound with peptone having a salmon, pink, or red color. This is a positive *Voges-Proskauer reaction*. The *Escherichia* never form acetyl-methyl-carbinol.

A recently described method\* for detecting the ability of bacteria to form this substance introduces a new principle in bacteriological technic. Here, instead of waiting several days for large numbers of bacteria to accumulate in the culture and to act upon the substrate, a very large, active, young, bacterial population *already formed* is added to the dextrose broth and only six hours are necessary for these billions of active cells to produce sufficient acetyl-methyl-carbinol to give a strong test. The method is of interest not only because of the saving in time but because it may be applicable to many tests of a similar nature with other species and other substances, for example, nitrate reduction, hydrolysis of various compounds, etc.

**Methyl-red Test** (*Clark and Lubs Reaction*).—As mentioned above, both *Escherichia* and *Aerobacter* produce acids and gases when dextrose is fermented. *Escherichia* cultures remain strongly

\* **Rapid test for acetyl-methyl-carbinol production** (*Voges-Proskauer Test*)  
Coblentz, J. M.<sup>19</sup>

Inoculate 5 ml. of 1 percent dextrose infusion-broth with a massive inoculum (2 to 3 mm.) from a 12-hour infusion-agar slant of the strain to be tested. Incubate at 30° C. for 6 hours. (Satisfactory results have also been obtained at 37° C.) At end of the incubation transfer 2 ml. of the broth culture into a culture tube 18 mm. in diameter and add 0.6 ml. of alphanaphthol solution (5 gm. of alphanaphthol in 100 ml. 95 percent ethyl alcohol). Shake slightly to mix and add 0.2 ml. of 40 percent KOH to which has been added 0.3 percent creatine. Shake the tubes vigorously for 30 seconds to 1 minute.

A positive reaction is characterized by an intense pink-rose coloration developing in a few seconds to ten minutes after reagents are added.

acid for several days and methyl red (an acid-alkali indicator) takes a definite red color (indicating acid) when added to the culture after four or five days' incubation. This is a *positive methyl-red test*. The *Aerobacter*, on the other hand, are able to attack the acids which they form from the dextrose, converting them, after four or five days, into carbonates or other nonacid substances. The medium is then found to have reverted toward a neutral or alkaline reaction and gives a yellow color (indicating relative alkalinity) when methyl red is added. This is a *negative methyl-red test*.

There are many other tests for differentiation between *Escherichia* and *Aerobacter*. For example, *Aerobacter* can attack substances,

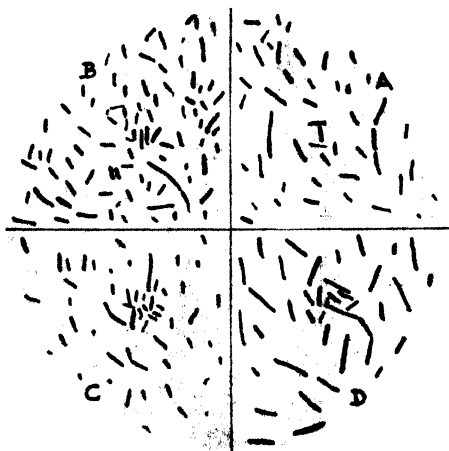


Fig. 203.—Four common species of soil bacteria: A, *Chromobacterium violaceum*, B, *Achromobacter liquefaciens*; C, *Flavobacterium aquatile*; D, *Alcaligenes faecalis* ( $\times 900$ ).

such as sodium hippurate, cellobiose and various organic acids, which *Escherichia* cannot metabolize.

An interesting group of related organisms, called *paracolon bacteria*, is discussed more fully in the section on enteric disease (see page 487). The paracolon species <sup>20, 21, 22, 23</sup> resemble the *Aerobacter* and *Escherichia* in most respects but differ especially in very slow fermentation of lactose and in virulence.

**Genera *Alcaligenes*, etc.**—These organisms all resemble the *Escherichia*, *Pseudomonas*, etc., in being gram-negative, non-spore-forming rods, growing well on ordinary laboratory media. Members of the genus *Alcaligenes* (especially *Al. faecalis*) are sometimes found in the human intestinal canal, possibly associated with path-

ological conditions there. They also occur in soil and water.<sup>23a</sup> They are closely related to the genus *Rhizobium*. In the soil, along with these genera, similar species of the saprophytic genera *Achromobacter*, *Flavobacterium* and *Chromobacterium* are found. The organisms of all these genera resemble each other morphologically (Fig. 203) and in lacking acid-producing powers (although often utilizing simple sugars without acid formation). Some are proteolytic and many are motile. Occasional species give trouble in the dairy by causing spoilage of ice cream (*Achr. lipidis*), or "ropy" milk or cream (*Al. viscosus*, Fig. 204).

*Achromobacter liquefaciens*, the type species of the genus named, is not pigmented, nor is *Alcaligenes faecalis*, type species of that genus.

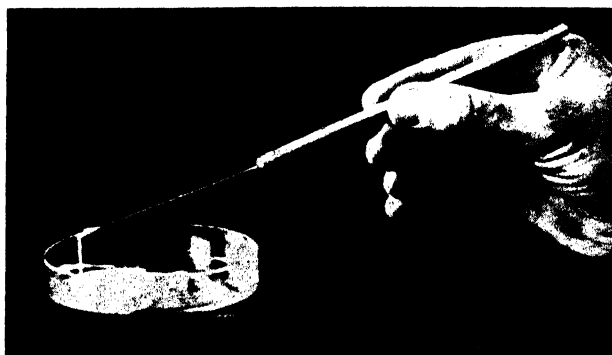


Fig. 204.—Ropy cream. Causative organism *Alcaligenes viscosus*. (Reprinted by permission from Hammer, "Dairy Bacteriology," John Wiley & Sons, Inc., publishers.)

*Chromobacterium violaceum*, the type species of the genus *Chromobacterium*, is of interest because of its violet pigment, while *Flavobacterium aquatile*, the type species of the genus, is characterized by a yellow pigment. *Fl. synxanthum* sometimes grows in milk at about 30° C., giving it a yellow color ("yellow milk") and is therefore of interest to dairy bacteriologists. In general, however, these genera are of lesser importance.

**Sanitation of Drinking Water.**—The diseases most frequently transmitted by drinking water are typhoid fever and cholera. They are diseases of the intestine and are transmitted by any material, especially milk, water, or food, contaminated with infected feces.

Bodies of water are convenient places for the disposal of sewage and other refuse and it was not uncommon, until even as late as

the latter part of the nineteenth century, for a city to pour its sewage into a body of water at one point and build the intake for a piped water supply not far away. Consequently, sewage got into the drinking water and epidemics of intestinal infection, especially typhoid and cholera, occurred.

Springs, streams, and lakes also become polluted through the drainage or seepage into them of infected surface washings. Today, we know how to prevent pollution of small bodies of water used for

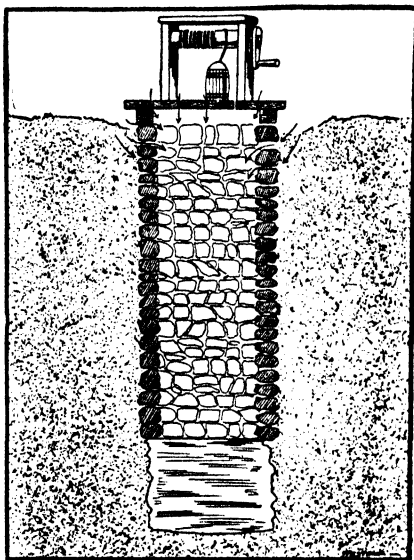


Fig. 205.—Usual method of pollution and even infection of well. The surface washings may contain the feces of a careless typhoid carrier who has defecated nearby. Such instances are numerous. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

drinking. Figures 205, 206, 207 and 208 show how wells may become polluted and how to construct wells to avoid pollution.

In dealing with large bodies of water, however, pollution is difficult to avoid at all times. The result is that such waters, if used for city water supplies, must be treated so that even occasional pollution with its dangers of infection may be guarded against.<sup>16</sup> The water is therefore collected in reservoirs and then piped to some sort of plant where it is usually filtered, especially if not perfectly clear and clean all the year round. In any case, it must always be disin-

fectured with chlorine, or live typhoid bacilli may at some time get into the water pipes and kill hundreds of people. This has happened repeatedly in the past. The small amount of chlorine necessary for this purpose is absolutely harmless even though it may give a "chemical flavor" to the water.

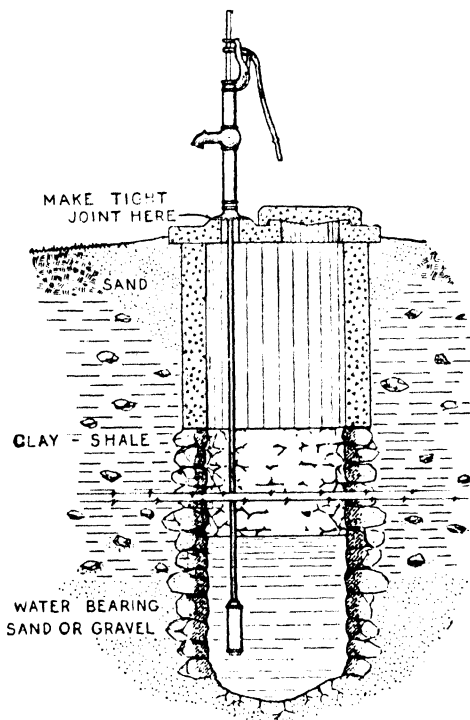


Fig. 206.—Showing how a well can be protected against pollution. Note the drainage away from the well and the water-tight curb and platform. (Minn. State Board of Health.)

**Filter Plants.**—*The Slow Sand Filter.*—Filter plants are of two main types: the slow sand filter and the rapid sand filter. Both imitate natural processes of water purification by allowing the water to seep through clean soil, or sand, the water collecting in under-drains and being piped to cisterns where it receives a dose of chlorine.

In the *slow sand process*, large sand and gravel beds are built up over drain pipes, starting with coarse gravel at the bottom and



graduating in size to rather fine sand at the top (Fig. 209). The water is led onto the sand and allowed to trickle slowly through.

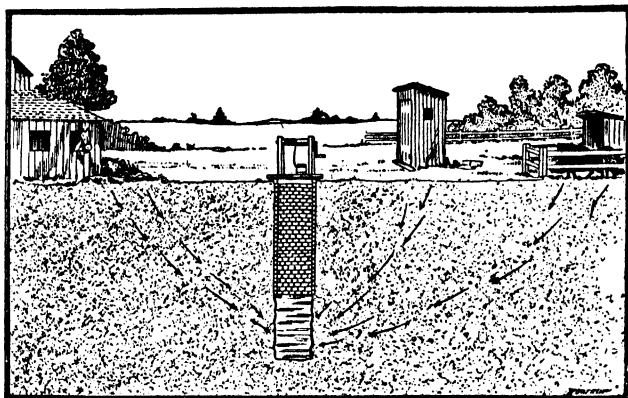


Fig. 207.—Popular idea of how wells become infected from surface pollution. This probably rarely takes place in rural districts, as the soil can usually hold back most of the impurities. The danger is great, however, where fissures, cracks, or crevices exist, or where sewage enters beneath the surface of the soil from broken drains or leaky privies, especially in limestone formations. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

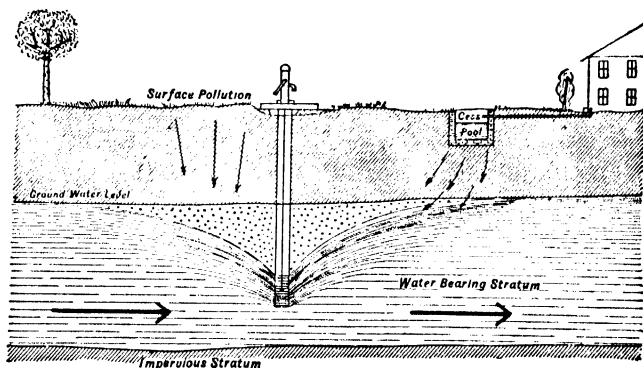


Fig. 208.—Depression of the ground water level by pumping and tendency to draw nearby pollution from the soil or cesspool against the normal direction of flow. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

The area of the slow sand filters is necessarily large because the water passes slowly through it. As filtration proceeds, day after day, there accumulates, especially in the upper layer of sand, a slimy

or gelatinous film composed of millions of bacteria and protozoa, which slowly closes up the pores in the sand. This makes the filter bed more and more effective but also causes the rate of filtration to become slower and slower.

This film is called the "Schmutzdecke" (German for "dirt-cover"). Through the action of enzymes, biological oxidation and reduction processes, and the ingestion of bacteria by myriads of protozoa inhabiting the slimy film, the bacterial and chemical quality of the water is greatly improved, the number of bacteria present in the filtered water becoming smaller and smaller as the action of the Schmutzdecke increases. When it finally becomes too thick,

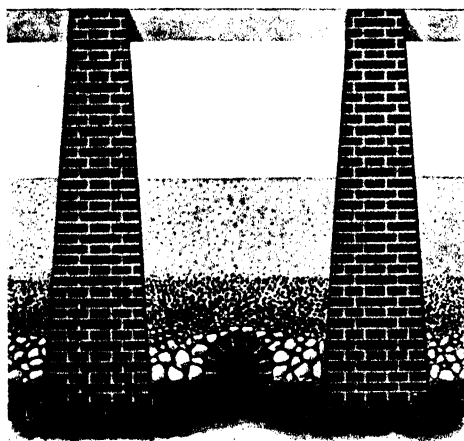


Fig. 209.—A slow sand filter. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Book Co., Inc., publishers.)

the filter is thrown out of service and the Schmutzdecke removed. A newly cleaned filter is not highly effective until the Schmutzdecke begins to form. The effectiveness of the filtration is constantly tested by bacteriologists in the plant who determine the numbers and kinds of bacteria present in the water during different stages of the filtration process, as well as in the finished product.

*The Rapid Sand Filter.*—The rapid sand filter is very similar to the slow sand filter in principle, but its area is much less and it does not depend on the growth of a Schmutzdecke. It filters water much more rapidly per unit of filter-bed area. In order to obtain rapid filtration of the water it is necessary that most of the foreign material in the water be first removed by some other means. This is

usually accomplished by *settling* and *coagulation*. Coagulative substances, usually ammonium aluminum sulfate, or a mixture of ferrous sulfate and lime, are added to the water. These form bulky, sticky precipitates which *adsorb* or *occlude* silt, bacteria and coloring matter. The water is held in large settling basins till most of the dirty precipitate has settled out and it is then run onto the filters. The particles to be removed consist chiefly of the floc remaining after the settling process and, being large, they are readily filtered

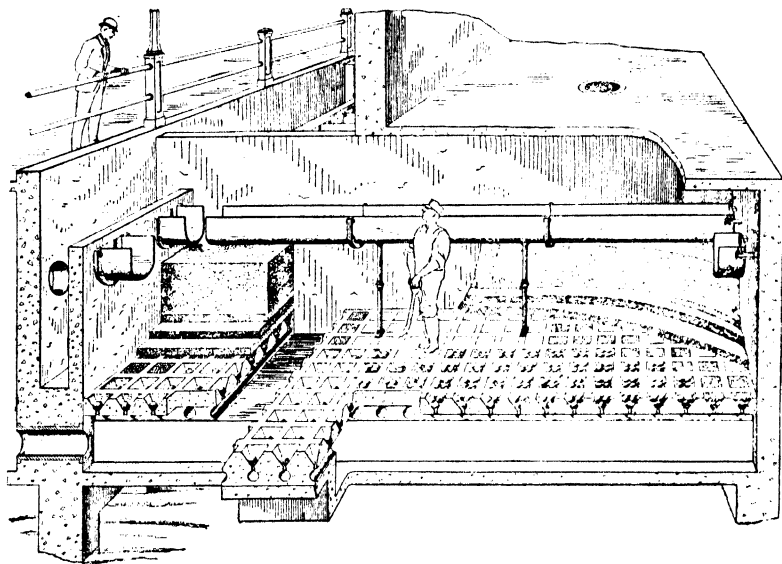


Fig. 210.—A rapid sand filter. Diagram showing, in rear corners, the different layers of sand; under foot, the drains for filtered water and the pipes admitting wash water in a reverse direction; at the digger's shoulders, the troughs leading away the overflow from the washing process. (From Horwood, "The Sanitation of Water Supplies," courtesy of Charles C Thomas, Springfield, Illinois, publishers.)

out by the sand. The filters remain in service for several hours or days, and are then washed by a flow of water and air forced upward through them. Figure 210 shows a rapid sand filter. After filtration the water is treated with chlorine and stored in underground cisterns. Highly polluted waters may be made potable, crystal clear and almost sterile by these devices, and it is due largely to these, as well as to improved methods of sewage disposal, that typhoid fever and cholera remain at a low level or absent in cities with water filtration plants.

**Sewage.**—The purification of sewage in cesspools and city sewage disposal plants is wholly dependent on biological processes in which protozoa, algae and putrefactive bacteria play the chief parts. The processes are, fundamentally, the same as those occurring in natural decay and putrefaction, but are controlled and exploited to the best advantage by the sanitary engineer.<sup>16, 24, 24a</sup> Many of the species of soil and intestinal bacteria already mentioned are involved in both processes.

The flora of sewage varies greatly, depending on its age and method of disposal. Fresh city sewage contains dilute feces, along with other city wastes. Consequently it contains the flora of the intestinal tract. This includes many strict anaerobes and facultative anaerobes, mostly saprophytic and heterotrophic. Common types from soil and intestine are *Escherichia coli*, and *E. freundii*, *Streptococcus faecalis*, *Clostridium perfringens*, and *Bacteroides vulgatus*, *Aerobacter*, *Proteus*, *Pseudomonas*, *Cytophaga*, *Thiobacteriales*, *Thiobacillus*, also yeasts, molds and actinomyces.

The saprophytic bacteria actively decompose the organic matter and produce their usual by-products,  $H_2S$ ,  $NH_4OH$ ,  $CO_2$ ,  $CH_4$ , lactic, butyric and acetic acids, alcohols, mercaptans, etc. If the process of sewage disposal is conducted in deep tanks, anaerobic decomposition proceeds best, and the facultative bacteria and the clostridia and other strict anaerobes thrive. In aerobic processes (see activated sludge, page 450) aerobic species predominate. As conditions of pH, O-R tension, type of organic matter and types of antagonistic bacteria change, the nature of the flora alters.

**Sewage Disposal.**—Of great importance in the prevention of water-borne disease is the proper disposal of sewage. Towns situated on streams below cities which pour raw sewage into the stream are certain to be constantly visited by typhoid fever unless protected by an efficient filter plant, and this has been the experience of many communities in the past. Such towns can sometimes force the city to install a sewage "purification" plant.

The sewage of a city is composed largely of water, and is handled in pumps and tanks much as is drinking water. Sewage disposal plants are operated to accomplish several ends as follows:

1. *Screening*; to remove bulky foreign matter such as bottles, paper, wooden boxes and other extraneous refuse.

2. *Separation by gravity* of all matter in the sewage which will settle to the bottom of tanks of various kinds.

3. *Aeration and biological treatment* of the supernatant fluid and possibly final *chlorination* of the aerated fluid.

4. *Digestion, and drying of the sludge or sediment.*

5. *Disposal of the sludge.*

Numerous methods have been devised for accomplishing these various ends. The first is largely a matter of mechanics and does not concern us. The second is of interest since it is here that bacterial action first comes into play. Various kinds of tanks are employed, all designed to allow the solid matter to settle out as much as possible, and in which the sewage is held for some time (twelve to thirty hours) in order to permit anaerobic bacterial decomposition of the organic matter to occur (Fig. 211). Such tanks are called

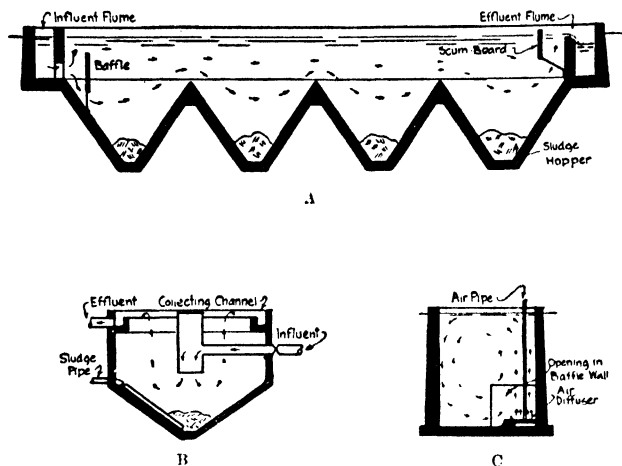


Fig. 211.—A Plain sedimentation tank, horizontal flow; B plain sedimentation tank, vertical flow; C activated sludge tank, spiral flow. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

sedimentation or "septic" tanks. Anaerobic and facultative denitrifying bacteria, proteolytic, fermentative, cellulose-digesting and other types of organisms, protozoan as well as bacterial, all play a rôle, and most of the organic matter is turned into liquids, gases and soluble materials, greatly reducing the volume of solid matter to be handled and making it much less offensive to the senses and much more stable biochemically. Sedimentation is sometimes hastened by the addition of flocculating agents similar to those used in water purification. The digested sludge is drawn off from the depths of the tanks through pipes.

**"Two-story" Tanks.**—Some tanks are made in two compartments, an upper and a lower. The upper portion is like a V-shaped trough

and serves to introduce fresh sewage. The solid matter settles out through a slot at the bottom of the V into a deep sludge sump as

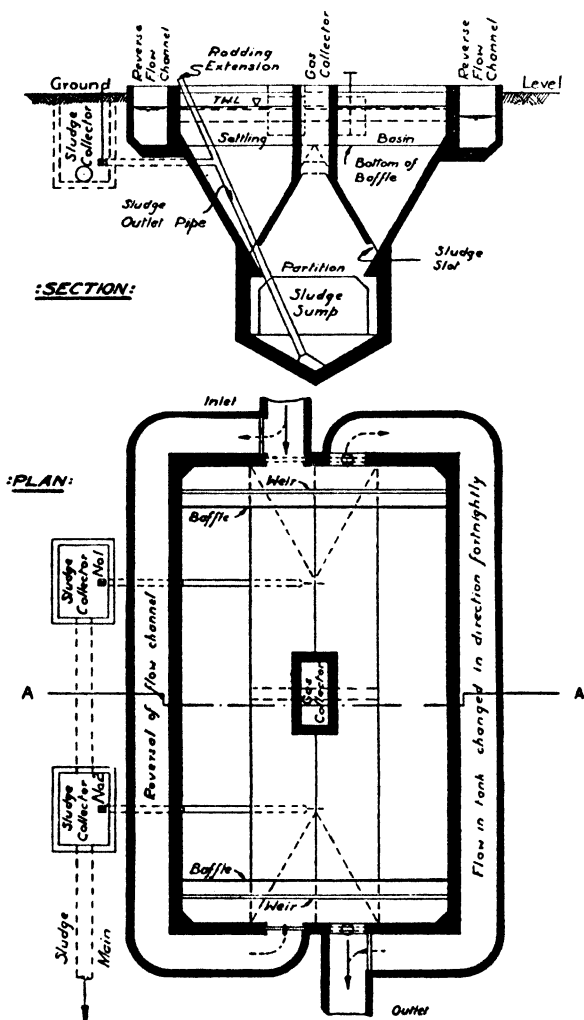


Fig. 212.—The Imhoff type of "two-story" tank. (T. P. Francis, "Modern Sewage Treatment," The Contractors' Record, Ltd., publishers.)

the fluid flows laterally along the trough. The solid matter, after settling to the lower compartment, is held and stored, digestion

and decomposition going on there. Gases from the sludge compartment are collected and led to the surface by a funnel-shaped structure inverted over the sump. The digested sludge, largely mineral residue, is pumped out from the bottom at intervals. The best known tank of this type is the *Imhoff tank* (Fig. 212).

*Aeration and Disposal of Fluid.*—The fluid part of the sewage after passage through the sedimentation tanks or above the sludge compartment of the Imhoff tank still contains much putrescible organic matter. This fluid is often disposed of, where a porous and dry soil is available, by surface ditches or by a subsurface irrigation system of tile pipes, furnishing excellent fertilizer for farm crops raised on the land. There is no danger of infection as typhoid, dysentery, and cholera organisms are killed either by the antagonistic action of saprophytic bacteria in the settling tanks or soil, or are filtered out by the soil and die.

The fluid may also be allowed to drain through artificial beds of sand, the filtrate being collected by under-drains much as in slow sand filtration of water. This process of purification is similar to those used in filtering drinking water, the sand grains becoming coated with a living film of protozoa and bacteria which feed upon the organic matter of the sewage and upon each other, the result being a much less putrescible liquid. Such filters require aeration daily to allow oxidation processes to occur and are therefore *intermittent* in operation. They must be cleaned at intervals of weeks or months.

The beds of sand may be replaced by deep tanks full of coke or broken stone. The tanks are filled with the fluid and allowed to stand so for some hours while the coke or stones, covered with films of living organisms, act like a catalyst, adsorbing and decomposing the organic matter in contact with them. These *contact tanks*, like the intermittent sand filters, must be drained and aerated daily to allow oxidation of the adsorbed matter to occur.

Another type of aerating device, operating on similar principles, is the trickling filter in which the fluid is intermittently sprayed over the stones instead of flooding them (Fig. 213).

The tank processes described in the foregoing paragraphs are largely dependent on anaerobic bacterial action. As we have seen, aerobic decomposition is often more rapid and complete and would be useful in sewage treatment. This can be accomplished by the *activated sludge* process.

*Activated Sludge.*—"If sewage standing in a tank is brought into intimate contact with air so that aerobic conditions are maintained

throughout the liquid, the particles of suspended matter after a time flocculate into masses of sludge swarming with microscopic life and capable of oxidizing organic matter readily. This sludge is known as 'activated sludge.' After this condition has been reached, sewage can be passed through the tank and a surprising clarification, nitrification, and reduction in bacterial content is obtained, providing aerobic conditions are maintained and sufficient quantities of activated sludge are intimately mixed with the traveling sewage. The bacterial reduction lies between 90 and 98 percent. In principle the process resembles that of aeration and filtration, the sand grains or stones of sewage filters being replaced by sludge particles suspended in the liquid."<sup>25</sup> In the section on Chlamydo-bacteriales it has been shown how one of those organisms causes interference—"bulking"—in activated sludge (see page 388).

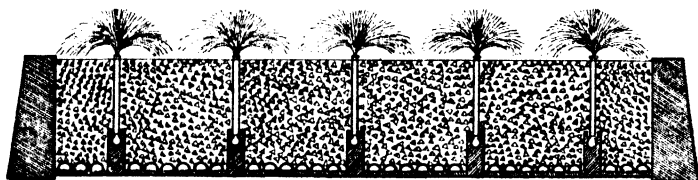


Fig. 213.—Typical section of a trickling filter. (Gordon M. Fair, Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

Disease transmission by water in the absence of elaborate filtration and chlorination systems is easily prevented. One needs only to bear in mind that all of the common intestinal pathogens are readily killed by exposure to 70° C. for ten to fifteen minutes and by contact with chlorine and other disinfectants. Knowing this, one can easily guard against these organisms by drinking only boiled water. This fact makes sabotage of water supplies by enemy agents in time of war a rather futile activity. Water for camp use may be treated with chlorine by using one of the methods of field chlorination involving tablets of  $\text{CaOCl}_2$  available on the market. A teaspoonful of fresh chloride of lime in 25 to 50 gallons of water will prove an adequate safeguard unless the water is very heavily polluted or dirty. At least an hour must be allowed for the chlorine to act.

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are blown about by the wind. Microorganisms are more numerous in air in dry weather than just after a rain.

Obviously only species resistant to desiccation and exposure to sunlight can persist long in the air. Species of bacteria commonly found are spore-bearing bacilli like *Bacillus subtilis*, micrococci like *Sarcina lutea*, and *Micrococcus luteus*, nonpathogenic corynebacteria, often called diphtheroids, chromogenic and nonchromogenic non-sporeforming rods like *Serratia marcescens* and its allies the *Chromobacterium* and *Flavobacterium*, and the spores of yeasts and molds. *Actinomyces* are also often found.

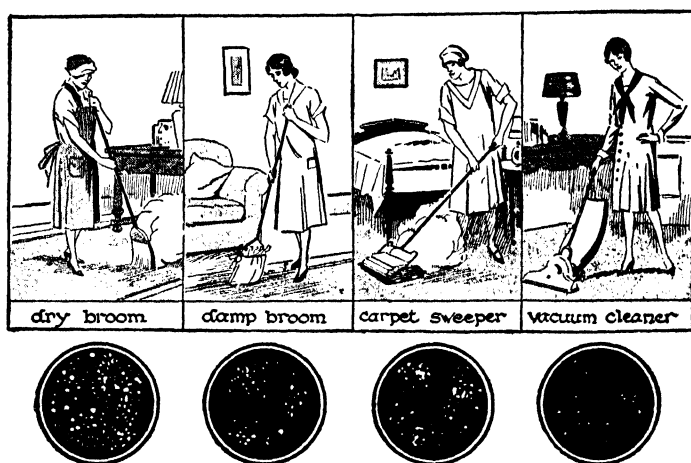


Fig. 214.—The colonies on the Petri dishes indicate the relative numbers of bacteria in the air during various methods of cleaning. (Hunter and Whitman, "Problems in General Science," American Book Co., publishers.)

In the dust and air of theaters, schools, hospital wards or the rooms of persons suffering from infectious bacterial diseases such organisms as tubercle bacilli, streptococci and pneumococci have been demonstrated, as well as other bacteria such as diphtheroids and staphylococci common in the normal mouth and throat. These are dispersed through the air by coughing, sneezing, talking and laughing (Fig. 138). Viruses may also be transmitted by air.<sup>2, 3, 4, 5</sup> The kinds of organisms present in air obviously depend on what sources of bacteria are being stirred up in the vicinity (Fig. 214) and on the direction from which air currents are flowing as well as their velocity. If the atmosphere is undisturbed, all dust will settle

and the air becomes sterile (see Tyndall's experiment on spontaneous generation, page 19).

**The bacteria of the upper air** have been collected by means of aeroplanes or other aerial devices (Fig. 215). For the collection of bacteria in the upper layers of the atmosphere, special apparatus must be used which excludes any possibility of contamination of the culture material, or other collection device, by dust from the airship or its occupants if such be used.<sup>6, 7</sup> A very interesting early study of bacteria of the air was made with the assistance of the

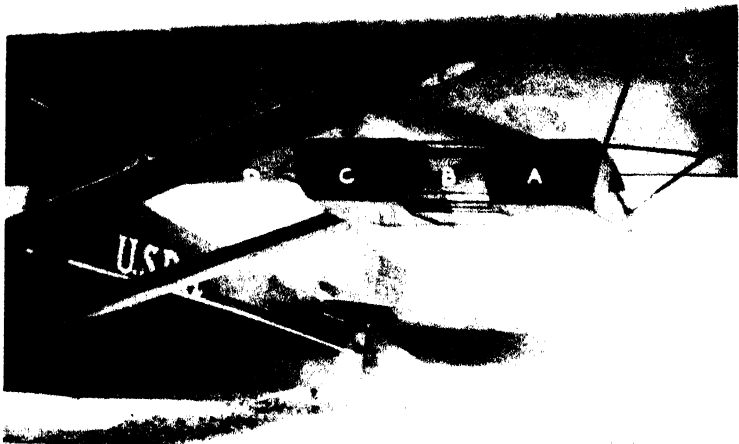


Fig. 215.—An airplane insect trap attached to wing of monoplane. A battery of fine trays is placed in compartment *A*, then pulled for exposure into area *B*; after exposing a screen tray it is then pulled into compartment *C*. All screen trays are pulled for exposure by wires (*D*), which are controlled from the cabin. Agar plates or sticky slides may be substituted for the screens to catch dust and bacteria. (From Tech. Bull. No. 673, U. S. Dept. Agr.)

famous aviator, Charles A. Lindbergh. "The flier collected these tiny forms of life at many points high over northern waters during the flight he and Mrs. Lindbergh made across the North Atlantic in the summer of 1933. The collections were made in cooperation with Fred C. Meier, of the Department of Agriculture, who planned the work . . . both disease organisms and others that are beneficial can be transported alive over long distances by air currents high above any ground barriers. Some of them cause diseases attacking crops and forests and possibly also some diseases of animals and humans. . . . On one slide, exposed far north of the Arctic Circle,

Mr. Meier was able to discover under the microscope more than forty different types of objects in a space of 5 cm. square" (*Associated Press*).

**Enumeration of Bacteria in the Air.**—Aerial organisms may be caught for study by exposing agar plates in any room for half an hour or so (depending on the amount of dust and disturbance present) and then incubating the plate. This method is more of a qualitative method than quantitative one, but gives a rough idea of the number and types of bacteria present in a given atmosphere. Like similar methods used in counting bacteria in milk and water, it is incomplete because some of the bacteria present will not grow under the cultural conditions provided. Special media may be used for the enumeration of certain kinds of bacteria, for example, blood agar for hemolytic species.

The numbers of bacteria present in air may also be determined by pumping a known volume of air through a small amount of sterile sand held in a tube through which the air passes, the sand acting as a filter to withhold the bacteria and dust (Fig. 216). The sand is then washed with a known volume of water and the numbers of bacteria present determined by putting dilutions of the wash water into plates with agar and counting the colonies that develop after suitable incubation. From these counts the number of bacteria per cubic meter of air may be estimated. Colonies may also be fished for identification of the species present.<sup>8</sup>

**The Air Centrifuge.**—The study of air pollution and the bacteria in air has been advanced by the use of the Wells centrifuge

(Fig. 217).<sup>9</sup> This is a device for causing a whirling current of dust-laden air to pass through a rapidly rotating cylinder lined with a film of nutrient agar, blood agar, or any medium appropriate to the bacteria to be obtained from the air and dust. The infected dust particles cling to the sticky agar film and the cylinder is eventually closed and incubated. Colonies may then be counted and fished for identification and, with a knowledge of the volume of air passed

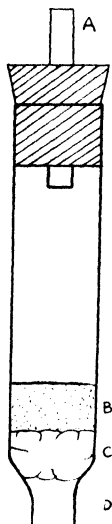


Fig. 216.—Tube for collecting dust from the air for bacteriological analysis. Air enters at A, deposits its dust on the sand (B), which is supported by a cotton plug (C), and leaves at D.

through the cylinder, the density of contamination of the air with each kind of bacterium may be estimated (Fig. 218).

A newer device<sup>10</sup> for collecting bacteria from air depends on the principle of atomization to produce a layer of liquid around each bacterial particle. The bacteria-laden mist is absorbed in a chamber containing a sterile mixture of broth and olive oil. The atomizing

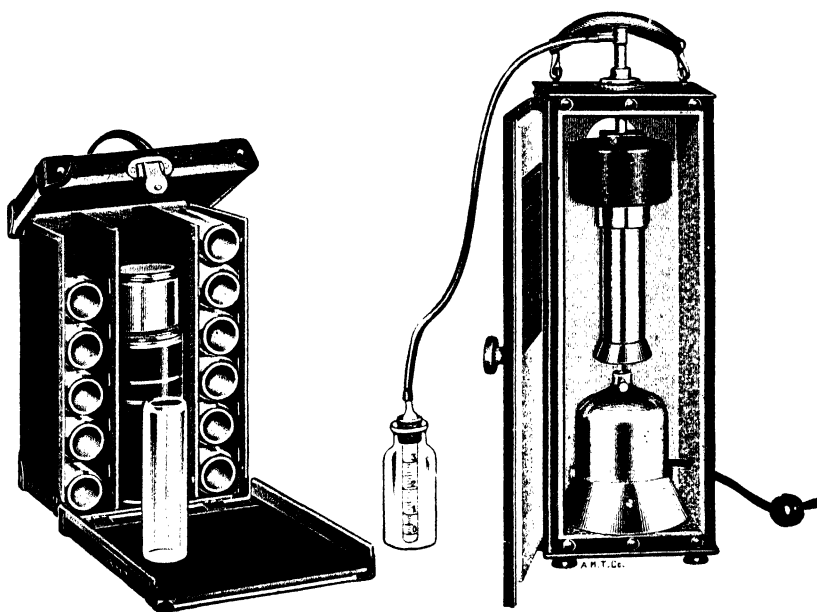


Fig. 217.—One form of air centrifuge (A. H. Thomas Co.). The apparatus at the right shows the driving motor at the base, the cylinder for catching the dust mounted vertically above, and at the top the blower-fan causing air to circulate through the cylinder. An air-flow meter with manometer is attached. At the left is shown a set of sterile cylinders ready for use in the whirling machine. Just before mounting each, a small amount of melted agar is introduced from the thermos bottle. The centrifugal force distributes the agar in a thin film on the inner surface of the cylinder where it soon "sets."

and collecting chambers are rinsed with the collecting fluid and plate cultures are made of measured quantities of the mixture. Certain corrections for loss by evaporation are applied. A flow meter measures the quantity of air sampled.

The collecting tube is shown in Figure 219. Air enters at E, passes through D and draws a mist of fluid through C. The droplets fall back into the fluid in the first chamber or are collected in the

hubbling chamber as they pass through B. The baffle and trap prevent any loss of fluid.

**Disinfection of Air.**—The early bacteriologists realized that microorganisms float about in the air attached to dust particles. Many of the living forms floating in air are spores of bacteria, molds and yeasts which may be harmless or may cause industrial spoilage or diseases of man or animals. In operating rooms air-borne bacteria are always a menace. In some manufacturing processes and in storage of foods, mold and bacterial growths are causes of

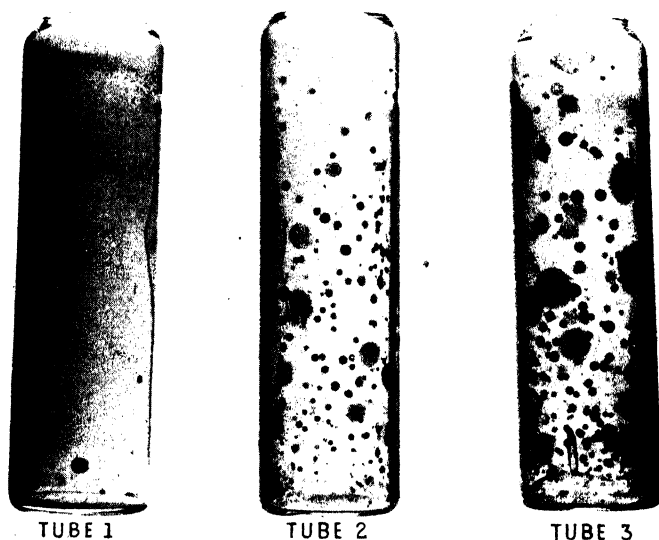


Fig. 218.—Bacterial colonies in air-centrifuge cylinders, showing different densities of bacteria in various parts of a hospital. Samples of air (1) premature ward, (2) and (3) children's wards. (Courtesy of Dr. C. F. McKhann.)

great loss. In hospital wards and institutions disease is often air-borne. The organisms are often transmitted by dust in the air, or as dried nuclei or droplets (see page 281). It is obvious that under such conditions disinfection or sterilization of the air is highly desirable. In recent years the problem has been given increasing attention and two general methods are now available for killing microorganisms in the air.

**Radiation.**—One is the irradiation of the air with *ultraviolet light* which, as pointed out in a previous chapter, is lethal to bac-

teria.<sup>11, 12, 13</sup> A radiation of wave length  $2537 \text{ \AA}$  is generally used as this is sufficiently bactericidal and at the same time not excessively irritating (see Fig. 220). Application of this is being made in schools, factories, storage warehouses, and hospitals. Ultraviolet-producing electric lamps are attached to the walls (Fig. 221), over-

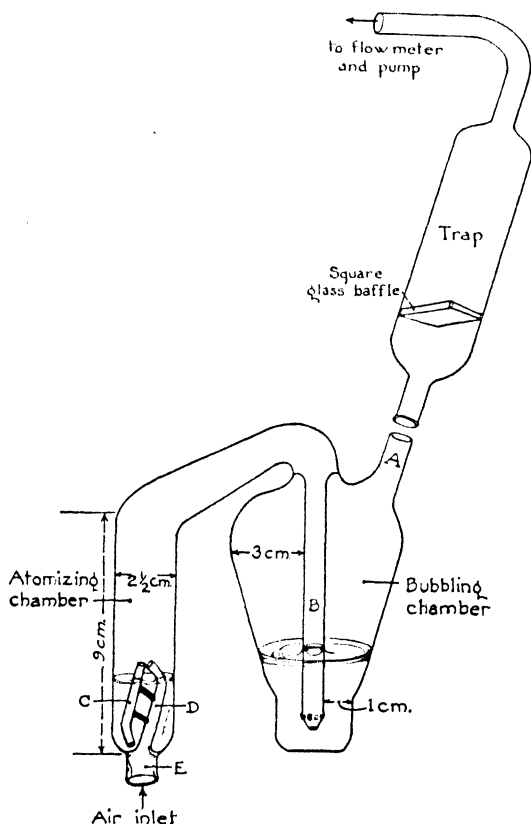


Fig. 219.—Device for collecting bacteria from air. (Moulton, Puck and Lemon—Science, Vol. 97.)

head (Fig. 222) or at strategic points between hospital beds or in corridors, etc. (Fig. 223). Deflectors are used to prevent direct exposure to the rays, which can cause serious "sunburn," and to protect the eyes.

**Aerosols.**—Another means of disinfecting air goes back to the



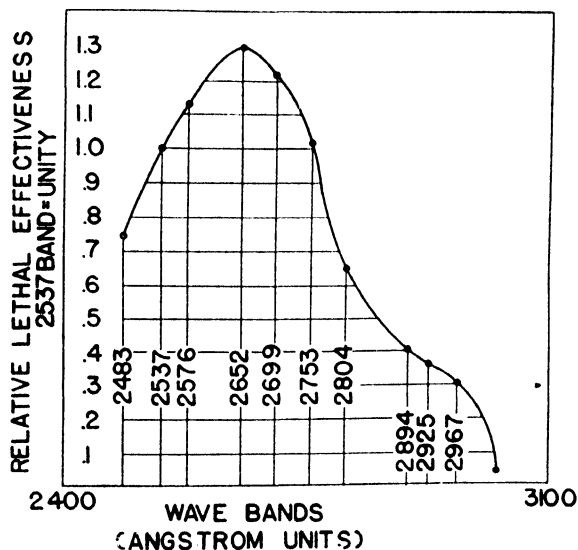


Fig. 220.—Relative bactericidal effectiveness of ultraviolet wave bands compared with waves of 2537 angstrom units. (Computed from data by Gates, after W. F. Wells, *Arch. of Phy. Therapy*, Vol. 23.)



Fig 221.—Use of ultraviolet radiation in a schoolroom. The irradiating lamps are seen at the top of the blackboard. The radiation reaches only the upper part of the atmosphere. Baffles prevent direct exposure of the lights to the children's eyes. (Courtesy of W. F. Wells.)

method used by Lister in his antiseptic surgery, and involves the use of sprays or very fine mists (aerosols) of bactericidal substances. Within the last two or three years the methods of using bactericidal aerosols have been greatly improved as a result of active research. Liquid aerosols consist of droplets which are about 1 to 2 microns in diameter. These droplets are small enough to remain suspended for long periods. Each droplet is concentrated disinfectant and therefore very effective. If sufficiently numerous they come into contact with all the bacteria in the air except under conditions of

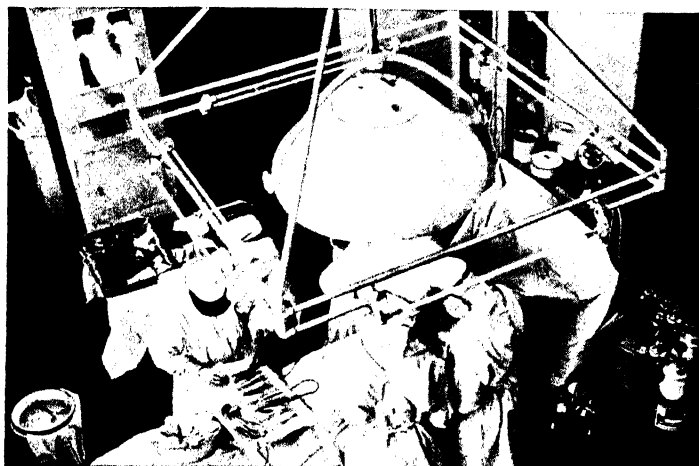


Fig. 222.—Maintenance of asepsis in surgery by means of bactericidal radiation from above. The large dome is for illumination. Ultraviolet light is given off by mercury vapor arcs in the rectangular frame of tubes. Note the eyeshades on the surgeons. (Courtesy of Westinghouse Electric & Manufacturing Co.)

very heavy contamination. Some substances are effective even in the gaseous state.

Various substances have been used as aerosols. They must not be toxic on prolonged inhalation, and must not vaporize readily but remain in the form of droplets. They should not be inflammable. Propylene glycol and triethylene glycol have been most investigated. They possess low toxicity, low surface tension (*i.e.*, are easily divided into fine mists), and good bactericidal activity. Approximately one part by volume of the disinfectant in 5 million volumes of air, *i.e.*, 1 gm. of disinfectant in 5 million cc. of air, is an effective concentration. An efficient atomizing and blowing ap-

paratus must be used to prevent the material from settling to the floor in large drops.<sup>14, 15</sup> The temperature and humidity of the air are important factors. If the air is cold and dry, or excessively humid, the effectiveness of aerosols is reduced. Relative humidities of around 40 percent at about 76° C. are favorable.<sup>16, 17, 18</sup>



Fig. 223.—Double cubicle in infants' ward. Ultraviolet light is directed downward from the long lamp near the ceiling extending from between the windows. This separates the cubicles. Two similar overhead lamps above the doorways send "curtains" of ultraviolet light down across the entrances to the cubicles. (Courtesy of W. F. Wells.)

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## CHAPTER 28

### PLANT DISEASES

IN PRECEDING chapters we have discussed bacteria of the soil, water and air, but dealt mainly with rather primitive organisms having, in general, autotrophic or rather simple nutritive requirements and, except for the genera *Escherichia* and *Rhizobium*, having little or no direct relation to higher plants or animals. In the present chapter we deal with very similar organisms, common in the soil, but which are capable of causing disease in a variety of plants. They are included in the genera *Phytomonas* and *Erwinia*.

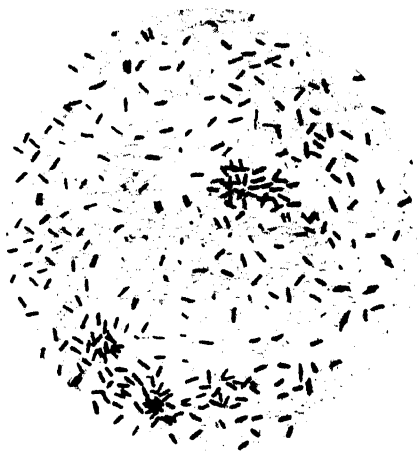


Fig. 224.—*Erwinia carotovora* ( $\times 900$ ).

These genera comprise, as at present classified, about 150 closely allied species of bacteria. The student contemplating this vast array of organisms need not, however, be unduly alarmed. Many of the species are obscure, poorly defined, rarely studied, and are not dealt with in detail here.

**General Characters.**—In general they are much like the bacteria common in water and soil discussed in the preceding chapters. Characteristics common to all of these organisms are: (1) a small, straight, cylindrical or rodlike structure, and dimensions usually not exceeding 10 microns in length and 0.5 to 1 micron in diameter (Figs. 224 and 225); (2) none of them forms spores; (3) all grow well on ordinary laboratory media such as extract or infusion agar

or broth, or on rather simple, mainly inorganic solutions in which the only source of energy or nitrogen is an organic compound; and (4) all are gram-negative and aerobic or facultative.

All of the species of the genus *Erwinia* and most of those of the genus *Phytomonas* are motile. Most species of *Phytomonas* and many of *Erwinia* form white or yellow pigmented, opaque colonies of a moist and butyrous (butter-like) consistency. In general they are metabolically active, fermenting numerous carbohydrates, often liquefying gelatin, and attacking a variety of other substances. In these respects, as well as others, they resemble the organisms of the genera *Pseudomonas*, *Escherichia* and *Aerobacter*.



Fig. 225.—*Phytomonas campestris* ( $\times 900$ ).

The *Phytomonas* and *Erwinia* were formerly grouped together in one tribe on the basis of their many similarities, including the property of producing diseases in plants. However, the genus *Erwinia* has been transferred to the intestinal group (family Enterobacteriaceae) because it possesses so many properties in common with the latter. It has recently been shown that the power to digest pectin, formerly thought to distinguish the Erwinae from closely similar intestinal species such as *Aerobacter aerogenes* and *Escherichia coli*, is common in these two species also, so that the differentiations between these genera appear to be rather slight and perhaps unnecessary.

The genus *Phytomonas* includes, all told, 137 or more species. These 137 species may be divided into three groups, the first of

which has the general properties given above, and is typified by *P. campestris*. The organisms of this group produce yellow pigment, are proteolytic, motile, and produce plant disease. In the second group are listed species very similar to the above, but most of which resemble organisms of the genus *Pseudomonas* more closely than *P. campestris et al.*, in producing a greenish or yellowish water-soluble pigment. Indeed, it has been found that *Pseudomonas aeruginosa*, a pathogen in man and lower animals, can also cause a rot-disease of plants. The third group includes species more like those of the genus *Rhizobium* in being metabolically rather inert but which differ in that, instead of stimulating benign nodules and living symbiotically, as in legumes, they cause plant tumors and other pathologic conditions. They also ferment carbohydrate with definite acid production.

The proper classification of such closely related genera as *Pseudomonas*, *Phytomonas*, *Rhizobium* and *Erwinia* is obviously an extremely difficult task on the basis of present knowledge. All are closely related. *Phytomonas tumefaciens* and *P. rhizogenes*, for example, produce nodular and other growths in the roots of plants, but fix no nitrogen as do the nodule-producing rhizobia. Certain strains of rhizobia resemble non-nodule-producing *Phytomonas* physiologically and in not producing nodules; others produce nodules but fix no nitrogen.

*Bacterium Radiobacter*.—One species occupying a rather anomalous position in this assemblage is *B. radiobacter*.<sup>1</sup> This so closely resembles the rhizobia and *P. tumefaciens* and *P. rhizogenes* in many respects that it is with difficulty distinguished from them. However it fails to produce nodules in, or to invade the roots of, leguminous or other plants with which it is commonly found in contact. Some of the differences and resemblances between *Rhizobium*, *B. radiobacter* and *A. aerogenes* and similar species are seen in the accompanying table (Table IV). *B. radiobacter* grows in very alkaline media (pH 11+), which is not usually the case with *Phytomonas* or *Rhizobium*. Some have classed it as a species of *Alcaligenes*.<sup>1a</sup>

#### PLANT DISEASES

That bacteria may infect plants, causing diseases which are highly injurious or fatal, was not generally understood until the careful work on this problem done by Erwin F. Smith.<sup>2</sup> The first name of this scientist has been given to the tribe (Erwineae) and to one of the genera of bacteria which are now known to cause plant diseases.

## PLANT DISEASES

Diseases in plants present several points of similarity to diseases of animals and man. First, they are specific. A given species of bacterium produces only one disease in a given plant while only certain species of plants are susceptible to any given disease. It is not difficult to imagine why some species of plants, like beans, for example, are not ordinarily subject to infection by the organism causing a disease like soft rot of carrots. The chemical and physical conditions favoring growth of the bacteria in the one are absent in the other. In some cases this is due to acidity of the plant sap, or the presence of inhibitory substances such as anthocyanin and tannin. An analogous host-parasite relationship is found in animal diseases. Horses, for example, never have typhoid fever, nor do birds suffer from swine plague.

The mechanism of resistance in plants, however, is more limited than that in animals. Animals have, in addition to the differences in chemical and physical make-up of species, *leukocytes*, and *antibodies* which circulate in the blood. Plants have neither leukocytes nor blood circulation and must depend on local resistance or species immunity. However, it has been shown that plants, parts of which have been affected by certain diseases, may produce immune outgrowths.

In many instances the bacterium causing a plant or animal disease can infect the plant or animal only through some definite natural *portal of entry*. In plants this may be the stomata\* of the leaves, the nectar ducts of the flowers, or an abrasion or wound. The intact outer bark and epidermal cells prevent the entrance of many parasites.

Some individuals of many species of plant seem to be more resistant than their fellows to invasion by the bacteria of a given disease and it has been found possible, by selecting and breeding these individuals, to develop resistant varieties. The same process doubtless occurs in nature and is known to function in animals.

**Armament of Plant Pathogens.**—It is against the local protections of plants that plant pathogens have special means of attack. Some of these pathogens, for example, the *Erwineae*, secrete enzymes (as pectinase) which digest the tissue-connecting substances of the plant (pectins, etc.), causing these to break down, so that the tissue cells, no longer cemented together by the pectin (middle lamellae), fall together into a slimy mass. The bacteria also produce enzymes capable of making available for their metabolism

\* Openings in the surfaces of the leaves through which pass oxygen, carbon dioxide and water vapor.



the plant cell walls, protoplasm and juices. Thus they are enabled to invade and multiply within the host plant, parasitizing it and destroying various parts, or so interfering with its growth and physiology that it is greatly injured or killed. All this is entirely analogous to animal and human disease.

**Types of Plant Disease.**—*Rots.*—Plant diseases vary in type, depending on the bacterial invader, on how and where the invading organism attacks, and on the anatomy and chemistry of the



Fig. 226.—*Erwinia carotovora*, streaked on raw carrot, which was for three days kept at 23° C. in a large covered dry culture dish. The inoculation was from a rotting raw potato which had been inoculated from a gelatin colony. The carrot was first washed, then soaked in 1 : 1000 mercuric chloride water, and cut with a cold sterile knife. The left (uninoculated) part remained sound. (Erwin F. Smith.)

plant. Those bacteria capable of digesting the pectins in tissues are likely to produce “rots” of various kinds; soft, hard, wet, dry, stem, root, and so on. *Erwinia carotovora* affects carrots and a number of other plants in this way (Fig. 226). Various species of *Erwinia* are most commonly involved as the cause of plant “rots.” *E. carotovora* is a very vigorous producer of pectinase.

Rots caused by true pathogens which attack and digest healthy growing tissues must be distinguished from spots of decay of dead

tissue due to common saprophytes of the soil, which start to grow in a part of the plant which has first been injured by some other means. These organisms produce ammonia or other poisonous substances which kill the plant tissues nearby, thus permitting further

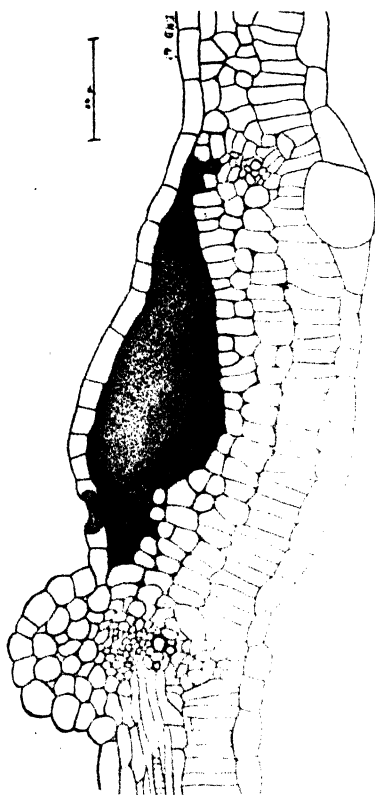


Fig. 227.—South African mulberry blight due to *Phytophthora mori*: section through a water-soaked spot on a leaf of the common mulberry which was fixed five days after inoculation, showing lower epidermis (left side) lifted, a large bacterial cavity (dark area) and penetration of the intercellular spaces of the mesophyll (center area) by the bacteria. (About 100  $\times$ .) (Ethel M. Doidge.)

growth and advance of the rot. *They do not initiate the rot. They are "opportunists."* Decay of dead tissue by common saprophytes of various species is also not a pathological process.

**Blights.**—Bacteria which invade the spaces between the cells

and grow in the plant juices are likely to cause discoloration or death of whole leaves or branches due to the stoppage of sap flow or to poisons (toxins) given off by the bacteria (Fig. 227). Such conditions are found in the "fire blights" of pears, apples and other pomaceous fruits (Fig. 228). *Erwinia amylovora* is one of the species causing such diseases, although various species of the *Phytomonas* are frequently responsible for this type of plant pathology.



Fig. 228.—Pear fruit showing effect of invasion by the fire-blight organism. The pearly beads on the surface are drops of exudate containing large numbers of bacteria. (After Jackson, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

*Wilts.*—Another type of disease due to invasion of the tissue spaces is exemplified by the so-called "wilt" of cucumbers, melons, squashes, etc., caused by the growth of *Erwinia tracheiphila*. The masses of bacteria become so dense in the sap vessels and spaces that flow of sap is obstructed and the plant wilts (Fig. 229). A wilt of corn is due to *Phytomonas stewarti*.<sup>3</sup>

*Spots.*—A number of plants are subject to a type of disease called *leaf spot* or "black spot." In such diseases the bacteria do not invade the tissues extensively, their destructive effect being



Fig. 229.—Young melon plants: No. 1, inoculated at X and Y with *Erwinia tracheiphila*; No. 2, healthy control. (E. F. Smith.)



Fig. 230.—Inoculated cotton leaves showing angular spots due to stomatal infection by *Phytomonas malvacearum*. Bacterial suspension sprayed on. Spots in second stage, i.e., beginning to shrivel and brown. Time, six weeks. One half natural size. (Erwin F. Smith.)

localized around their portals of entry, which are usually the stomata. The diseased area in some types of leaf spot drops out,

leaving a small "shot hole." "Leaf spot" of peaches or cotton is a good example of this type of disease (Fig. 230). The *Phytophthora* are chiefly involved in producing leaf spots in various plants. Blister spot of apples is caused by *Ph. syringae*.<sup>3a</sup>

**Abnormal Growths.**—A very common warty or cancer-like overgrowth of plant tissues, called *crown gall* or *root gall*, is caused by bacteria (Fig. 231). Galls occurring on a wide variety of plants



Fig. 231.—Crown gall on white Paris daisy (*Chrysanthemum frutescens*). Plant inoculated with a pure culture of *Phytophthora tumefaciens* and photographed seven months later. One branch killed. About one quarter natural size. (Erwin F. Smith.)

are due to *Phytophthora tumefaciens*.<sup>4</sup> The organisms grow in the tissues and irritate the cells so that an abnormal or pathological overgrowth is produced. This is very suggestive of human cancer, but differs from it in many fundamental respects. Erwin F. Smith advanced a number of reasons for believing that plant galls and human cancer are of similar nature, but students of cancer have not agreed with him. The cause of human cancer is not known.

Some very suggestive relations between plant neoplasms (tumors) and tumor-producing bacteria have been revealed by White at the Rockefeller Institute. Strains of the microorganisms cultivated in media containing glycine lose the power to produce plant tumors, but if the plant tissues are treated with certain growth-

stimulating substances (hormones), tumors develop when the bacteria are present, although neither bacteria nor hormone alone produce the neoplasms. In another series of experiments certain plants were inoculated with the organisms and then held for ten days at about 46° C. The bacteria seemed to be destroyed and no tumors developed at this high temperature. However, tumors developed when temperatures were lowered to normal, suggesting that the microorganisms had so altered the plant cells that they acquired the property of tumor formation in the absence of the living bacteria.<sup>5</sup>

**Transmission of Plant Diseases.**—The experimental plant pathologist accomplishes the infection of healthy plants with pure cultures of pathogenic bacteria by spraying the plant with a suspension of the desired organism or by pricking the plant, stem, root, leaf or seed with a needle carrying the bacteria in question. In nature, these operations are accomplished by wind and water carrying infected dust, pollen or parts of plants from one place to another. The bite or sting of insects which have just been feeding on diseased plants infects healthy ones. The bite of insects is also a means of transmission not uncommon in human disease as we shall point out later (yellow fever, malaria, bubonic plague, Rocky Mountain spotted fever). A farmer or horticulturist may, like a surgeon, also transfer pathogenic bacteria on pruning or other instruments unless he is careful to disinfect them. He may leave stumps of amputated branches open to infection unless he covers or disinfects them. Good paint, cresol, or bichloride solutions are useful for this purpose.

**Control of Plant Diseases.**—No universal rule can be laid down for the control of all plant disease; neither can we control all human or animal disease by any single procedure. However, some general precautions may be taken which may reduce the amount of damage and the spread of plant infections. Diseased plants or portions of them should be burned. In handling them, the utmost precaution must be taken not to allow them to come into contact with other susceptible plants. The soil around them may be disinfected if it is of small extent, or it may be allowed to remain fallow for a year or more. Instruments and gloves used in handling diseased plants should be disinfected.

Control of insect pests will reduce the transmission of many plant diseases. Eradication of weeds also may reduce disease, since these serve sometimes to harbor not only transmitting insects, but the disease organisms themselves. Analogous relations



exist between many lower animals and man. Animals serve as "reservoirs" of many human pathogens, such as *Salmonella* (rats, mice), *Brucella* (cattle, swine) and so on.

Spraying plants with Bordeaux mixture or lime-sulfur early in the season and at ten- to fifteen-day intervals during the summer often prevents the ravages of disease, especially fire-blight. Copper sulfate (the effective agent in Bordeaux mixture) and sulfur are effective general fungicides. These do not always cure the diseases, but usually prevent their development. Spraying, therefore, should be regarded as a prophylactic or preventive measure. Spraying is especially desirable during hot, damp weather.

Finally, as with human beings, sturdy, well nourished and well watered plants are more resistant to all sorts of parasites. Certain individual plants sometimes stand out unharmed in a whole field ruined by some disease. Such plants should be cared for and their seed preserved. In such manner naturally resistant varieties are propagated and often net their discoverers a great deal of satisfaction if not money.

An important and widely used means of preventing the spread of plant diseases from one field to another is the disinfection of seeds, bulbs or cuttings used for propagation. This is done by soaking them for short periods (2 to 6 hours) in solutions of disinfectants such as bichloride of mercury, or exposing them to formaldehyde vapors under a tarpaulin. The procedures vary for different crops and details will be found in books on agriculture and plant pathology.<sup>6-9</sup>

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## CHAPTER 29

### BACTERIA OF ENTERIC DISEASE

WE HAVE DEALT, in the preceding chapters, with groups of gram-negative, nonspore-forming, aerobic and facultative, rod-shaped bacteria commonly found in soil, water, the intestine and minor infections. We have seen that we could arrange certain

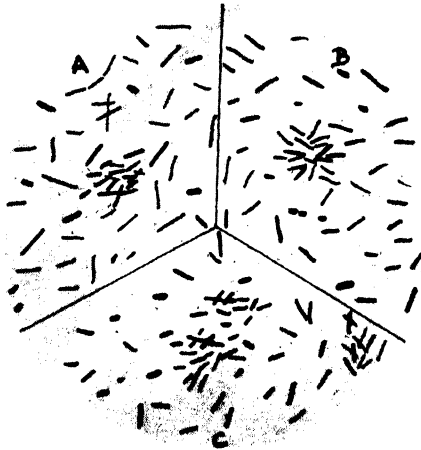
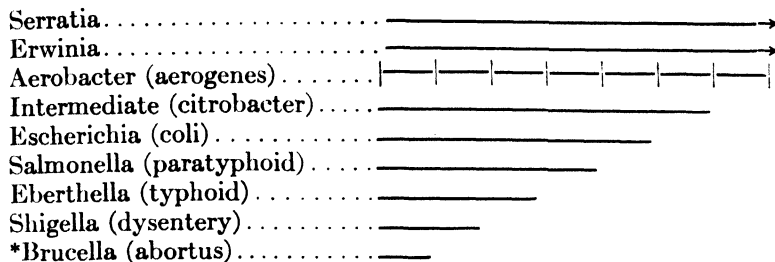


Fig. 232.—Various members of the tribe Salmonelleae. A, *Salmonella choleraesuis*; B, *Eberthella typhosa*; C, *Shigella paradysenteriae* ( $\times 900$ ).

genera of gram-negative species into a more or less regular series starting with entirely saprophytic forms and proceeding toward definitely parasitic forms—the former typified by such genera as *Serratia*—through the *Aerobacter* and *Escherichia* and ending with the three genera of dangerous pathogens which we shall discuss

in this chapter, namely *Salmonella*, *Eberthella* and *Shigella*\* (Fig. 232). Bacteria of these three genera cause enteric disease.

The idea of a metabolic gradient has been extended by Stuart<sup>1</sup> as shown in Fig. 233.



\* Now in family Parvobacteriaceae.

Fig. 233.—Metabolic “gradient” within the family *Enterobacteriaceae*. Divisions in the line opposite *Aerobacter* may be taken to represent in order, from left to right, the ability to ferment mono-saccharides, di-saccharides, tri-saccharides, to produce acetyl-methyl-carbinol, to liquefy gelatin, etc. In other genera, the shorter the line opposite the genus, the lower the metabolic activities of that genus. When specific substances such as particular carbohydrates are used in the base line, the gradient breaks down in a few places, but the general concept that we are dealing with different degrees of specialization among the several genera still holds true. (Stuart, New England Water Works J. 55:355, 1941.)

It is of interest and importance to note that, with the exception of certain dysentery strains, these three genera differ from all the previously discussed “gram-negative intestinal group” of organisms (*Escherichia*, *Proteus* and *Aerobacter*) in failing to ferment lactose and sucrose or to digest gelatin. Further, none produces acetyl-methyl-carbinol. Morphologically, and in most physiological respects, the members of these genera of pathogens closely resemble one another (Chart II). There is considerable immunological interrelationship between some species of each genus.

Typical members of the three genera are differentiated on the bases of motility, indol formation, and the production of gas from dextrose.

**Genus *Salmonella*.**—Of the salmonellas, *S. typhimurium*, *S. paratyphi* (also commonly spoken of as *Bacillus paratyphosus A*),

\* The apparently meaningless generic names are derived from names of famous bacteriologists. Salmon was an American scientist, noted for his work on hog cholera (1885). Eberth was a German who first discovered typhoid bacilli in 1880. Shiga was a Japanese bacteriologist who first determined the cause of highly fatal epidemics of dysentery in Japan in 1896.

## CHART II

## DIFFERENTIATION OF INTESTINAL SPECIES OF ENTEROBACTERIACEAE

Gas always predominantly CO <sub>2</sub>	$\left\{ \begin{array}{l} \text{Voges-Proskauer} + \\ \text{Methyl red} - \\ \text{Cellobiose} \oplus \\ \text{Citrate utilization} \\ \text{tests} + \end{array} \right.$	$\left\{ \begin{array}{l} \text{motile} \\ \text{gelatin} + \\ \text{not encapsulated} \end{array} \right.$	<i>Aero-</i> <i>bacter</i> <i>cloacae</i>
		$\left\{ \begin{array}{l} \text{nonmotile} \\ \text{gelatin} - \\ \text{encapsulated} \end{array} \right.$	<i>Aero-</i> <i>bacter</i> <i>aerog-</i> <i>enes</i>

D

c

x

l

r

o

s

e

⊕

## NONPATHOGENS

Gas always predominantly H<sub>2</sub>
 $\left\{ \begin{array}{l} \text{Voges-Proskauer} - \\ \text{Methyl red} + \\ \text{Motile} \end{array} \right.$ 
 $\left\{ \begin{array}{l} \text{citrate utilization} \\ \text{tests} + \\ \text{indol} - \\ \text{cellobiose} \oplus \end{array} \right.$  *Esch.*  
*freundii*  
("Citro-  
*bacter*")

 $\left\{ \begin{array}{l} \text{citrate utilization} \\ \text{tests} - \\ \text{indol} + \\ \text{cellobiose} - \end{array} \right.$  *Esch.*  
*coli*

LACTOSE ⊕

LACTOSE -

Acid and gas formed from dextrose; all motile	$\left\{ \begin{array}{l} \text{S} \oplus \\ \text{u} \\ \text{c} \\ \text{r} \\ \text{o} \\ \text{s} \\ \text{c} - \end{array} \right.$	gelatin + ; <i>Proteus vulgaris</i>	
		$\left\{ \begin{array}{l} \text{X} - \text{Salmonella} \\ \text{paratyphi} \end{array} \right.$	$\left\{ \begin{array}{l} \text{nitrates not reduced;} \\ \text{H}_2\text{S not formed; inositol} - \\ \text{H}_2\text{S produced} \end{array} \right.$
D e x + t r or ⊕ o s e	$\left\{ \begin{array}{l} \text{y} \\ \text{l} \\ \text{o} \\ \text{s} \\ \text{c} + \end{array} \right.$	$\left\{ \begin{array}{l} \text{S} - \text{S. suispestifer and S. choleraesuis} \\ \text{o} \\ \text{r} \\ \text{b} \end{array} \right.$	$\left\{ \begin{array}{l} \text{I} - \text{S. enteritidis and S. hirschfeldii} \\ \text{n} \\ \text{o} \\ \text{s} \end{array} \right.$
		$\left\{ \begin{array}{l} \text{i} \\ \text{t} \\ \text{o} \\ \text{l} + \end{array} \right.$	$\left\{ \begin{array}{l} \text{nitrates reduced;} \\ \text{H}_2\text{S produced} \\ \text{S. schottmülleri and} \\ \text{S. typhimurium} \end{array} \right.$

## PATHOGENS

Acid formed,  
but no gas,  
from dextrose
 $\left\{ \begin{array}{l} \text{M} - \text{Shigella} \\ \text{o} \\ \text{t} \\ \text{i} \\ \text{l} \\ \text{i} \\ \text{t} \\ \text{y} + \text{Eberthella typhosa} \end{array} \right.$ 
M - *S. dysenteriae*
 $\left\{ \begin{array}{l} \text{a} \\ \text{n} \\ \text{n} \\ \text{i} \\ \text{t} \\ \text{e} + \text{S. paradyenteriae} \\ \text{and others} \end{array} \right.$ 
 $\left\{ \begin{array}{l} \text{mannite} +; \\ \text{nitrates reduced;} \\ \text{H}_2\text{S produced} \end{array} \right.$ 

Note: + = The organism attacks, or produces a positive test, or produces a fermentation without gas. ⊕ = Fermentation with gas. - = Negative reaction.

*S. schottmülleri* (also commonly known as *Bacillus paratyphosus* B), *S. enteritidis* (also commonly known as Gärtner's bacillus or [incorrectly] the "food-poisoning bacillus") and several other closely related species such as *S. choleraesuis* (or the "hog-cholera bacillus"), are among the most important members of the so-called "paratyphoid group" or genus *Salmonella*.

Various biochemical properties such as ability to ferment various carbohydrates and production of hydrogen sulfide serve to distinguish the species in each genus, and serological (agglutination) tests are used in addition.

TABLE V  
DIFFERENTIAL FINDINGS, "ENTERIC DISEASE" ORGANISMS

Genus	Gas* from Dextrose	Motility	Indol
<i>Salmonella</i> . . . . .	+	+	—
<i>Eberthella</i> . . . . .	—	+	±
<i>Shigella</i> . . . . .	—	—	±

\* All ferment dextrose with acid formation.

**Antigenic Analysis.**—Indeed, the use of the agglutination test for the analysis of antigenic structure and classification is beautifully illustrated by the genus *Salmonella*. As stated in a previous chapter (see page 294), many closely related species of bacteria may contain one or more antigenic components in common. It was explained that agglutinating sera prepared by the injection of animals with such species often show much cross-agglutination with closely related species. By the process of dilution or agglutinin adsorption these sera can be deprived of virtually all but one type of agglutinin and with a group of such sera, representing all of the antigenic components in the genus, one may test any given organism for the presence of different antigenic components and assign to it a "formula" expressing the antigenic complex of which it is composed. This sort of antigenic analysis has been developed in the genus *Salmonella* to a high degree by such workers as Kauffmann, White, Edwards, Mickle and others, and we may now state the antigenic formula for each species, <sup>2, 3, 4, 5</sup>

Such antigenic analysis has served to reveal several interesting relationships and to confirm or refute, in some respects, differentiations formerly made almost entirely on biochemical grounds. For

example, the salmonellas are related to the eberthellas by the possession, in common, of certain antigens numbered IX and XII by both *E. typhosa* and *S. enteritidis*. Accurate antigenic analyses of strains of salmonellas responsible for outbreaks of food infection or other diseases in man or animals often yield data of great value to the epidemiologist and the health official.<sup>6, 7, 8</sup> In diagnostic work it is sometimes convenient to prepare group sera representative of several related species. For example, in one useful system group A serum agglutinates *S. paratyphi* and related species; Group B, *S. schottmülleri* var. Java and some other varieties; Group C serum, *S. choleraesuis* var. Kunzendorf, *S. amherstiana* and similar organisms, and so on (see Table VI). Individual specific sera may be used to differentiate related varieties in each group.

**Kauffmann-White Schema.**—The antigens referred to above as IX and XII occur in species of salmonellas which are members of a series often referred to as the “Kauffmann-White schema.” In this schema the O (somatic) antigens are given roman numbers and the flagellar or H antigens arabic numbers if of the *group* phase. If in the specific phase, the H antigens are given small roman letters. The terms *group phase* and *specific phase* refer to variations in the antigenic structure which occur in the flagella of some species. The antigenic structure of any given species may therefore be expressed in terms of these numbers and letters. For example, *S. typhimurium* has the antigenic formula IV, V, XII; b, i; 1, 2, 3. (See Table VI.)

TABLE VI  
ANTIGENIC FORMULAE OF SOME SALMONELLA SPECIES

Group	Species	O Antigens	H Antigens	
			Specific	Group
A	<i>Salmonella paratyphi</i> .....	I, II, XII	a	....
B	<i>Salmonella schottmülleri</i> .....	(I) IV, V, XII	b	1, 2
	<i>Salmonella typhimurium</i> .....		i	1, 2, 3
C	<i>Salmonella hirschfeldii</i> .....	VI, VII	c	1, 4, 5
	<i>Salmonella choleraesuis</i> ... ..		c	1, 3, 4, 5
D	<i>Salmonella enteritidis</i> .....	IX, XII	g, o, m	....
	<i>Salmonella pullorum</i> .....		....	....
	<i>Eberthella typhosa</i> ( <i>S. typhi</i> )....		d	....

Many antigenic types very closely resembling the above, differing perhaps in only one antigenic component, or one biochemical character, have been given species names. Some of these names are derived from the place where they were found, as, for example, "senftenburg," "newport," "*infantis*,"<sup>9</sup> "Mississippi."<sup>10</sup> The list of species is long.<sup>11</sup> The principal species are named in the table below. Their biochemical differentiation is usually not difficult (Table VII), although serological tests must be resorted to in some instances.

TABLE VII  
SOME BIOCHEMICAL CHARACTERS OF IMPORTANT SALMONELLA SPECIES

Species	Xylose	Arabinose	Maltose	Inositol	H <sub>2</sub> S Production
<i>Salmonella schottmülleri</i> .....	⊕*	⊕	⊕	⊕	+†
<i>Salmonella typhimurium</i> .....	⊕	⊕	⊕	⊕	+
<i>Salmonella hirschfeldii</i> .....	⊕	⊕	⊕	—	+
<i>Salmonella enteritidis</i> .....	⊕	⊕	⊕	—	+
<i>Salmonella pullorum</i> .....	⊕	⊕	—	—	+
<i>Salmonella paratyphi</i> .....	—	⊕	⊕	—	—
<i>Salmonella choleraesuis</i> .....	⊕	—	⊕	—	— or +

\*⊕ = acid and gas. †+ = H<sub>2</sub>S.

**Salmonella Infections.**—*Salmonella* organisms are not infrequently transmitted to human beings by milk and water supplies. Their classical and most common mode of transmission, however, is by means of food which has become infected and allowed to stand in a warm place, after little or no cooking, so that the organisms can grow. These organisms grow well at warm room temperatures (80° F.). Infection of the food is sometimes from a human carrier of *S. schottmülleri* or, probably more frequently, by contamination with the excrement of mice or rats which harbor particularly *S. typhimurium* and *S. enteritidis*. So-called "meat poisoning" results from eating raw or improperly cooked flesh of cattle, swine or other animals suffering from infection with these organisms, especially *S. choleraesuis* and *S. enteritidis*. *Salmonella* infections and intoxications from food are commonly (but incorrectly; see botulism, page 527) spoken of as a form of "food poisoning."

Obviously, avoidance of these diseases means cleanliness in the kitchen; sanitary habits on the part of food handlers; care to see that food is properly cooked to kill all organisms, even those in the center of large masses; proper refrigeration of stored food; and avoidance of uncooked foods at club suppers or on picnics, often prepared during the morning or previous evening and then unwittingly *incubated in the kitchen* or in transit. *Salmonella* food infections are very common.

The diseases caused by organisms of the *Salmonella* group are usually types of gastro-enteritis, prominent symptoms being fever, abdominal pain, nausea, vomiting, headache, diarrhea and general weakness coming on from ten to twenty-four hours after the infectious meal. Usually the outcome is not fatal but outbreaks differ in this respect. The acute type of food-borne disease is usually of short duration (a few hours to several days) depending on the species involved, the amount of toxin (?) ingested along with the organisms, and the resistance of the patient. Such attacks are usually due to *S. choleraesuis*, *S. typhimurium* or *S. enteritidis*. Prolonged, typhoid-like attacks are usually due to *S. paratyphi*, *S. schottmülleri* or *S. hirschfeldii*. *S. pullorum* causes "white diarrhea" in poultry. As shown by Seligmann, Saphira and Wassermann<sup>8</sup> many other conditions, as meningitis, pneumonia, etc., may be caused by *Salmonella* species.

Methods for laboratory diagnosis of these infections are similar in all respects to those used for typhoid fever (see page 488). Blood cultures are not so regularly successful, especially in the acute forms of salmonellosis, but should never be neglected. Spinal fluid cultures are not infrequently indicated, especially in children.<sup>8</sup>

**Genus Eberthella and Typhoid Fever.**—A number of non-pathogenic species of bacilli like *E. typhosa* are found in sewage and soil, but their true identity is obscure. *Eberthella typhosa*, the principal member of this genus, is like the pathogenic salmonellas and shigellas in causing gastro-enteritis and particularly an infection of the small intestine, typhoid fever. It differs in primarily invading the blood stream.

**Typhoid Fever.**—Our knowledge of typhoid fever serves to illustrate a number of important bacteriological principles and deserves rather detailed discussion. As is true of *Salmonella* infections and, indeed, of most of the gastro-intestinal diseases due to bacteria, typhoid fever may be transmitted by sewage-polluted water and milk, since, as we shall see presently, the bacilli leave the body in the intestinal discharges. The disease may, in fact, be

transmitted by any means which effect the transfer of infectious feces or urine to the mouth and so to the intestines of normal human beings. Of these means of transmission we must remember especially the four "F's": Feces, Fingers, Food and Flies.

Once the organisms (*Eberthella typhosa*) gain access to the susceptible individual in sufficient numbers, the body attempts to arrest their further progress by localizing them in small nodules of lymphoid tissue, called "Peyer's patches," in the lining of the small intestine—a sort of physiological concentration camp, as it were. But the invaders increase and escape into the blood stream and multiply everywhere. It is then, during the first five to seven days of the disease, that bacteriological diagnosis is attempted by means of blood cultures. The patient meanwhile has fever, abdominal distress and is generally ill. This is probably due in large part to the "O" and "Vi" substances in the typhoid bacilli, which are *endotoxic* carbohydrate-protein complexes. Localized foci of infection may persist in various organs, or in bone marrow.

After a day or more of enduring the presence of these foreign organisms in the blood, the body begins to react and develops a "corps" of antibodies specifically trained to cope with the situation. Agglutinins appear, directed against both the specific H (or flagellar) proteins of the bacilli and also against the somatic O (or group) antigens, as well as against the Vi (or "virulence antigen") present in virulent typhoid bacilli.\*<sup>12</sup>

The H, O and Vi antibodies increase in amount until, by the second week, they are easily demonstrable in the blood serum by test tube methods and furnish valuable means of diagnosis. At the same time protective antibodies appear, which can be demonstrated to protect mice against experimental infection with graded doses of virulent typhoid bacilli.<sup>13, 14</sup> These are not identical with H or O agglutinins but may be related to Vi agglutinins. The bacilli then find the blood too unfavorable and disappear. How-

\* The typhoid bacillus serves to illustrate a form of virulence which is possibly due to at least two factors, one responsible especially for its poisonous properties (the O component?), and another conferring aggressiveness upon it (the so-called Vi component?), neither being an *exotoxin*. Neither component *alone* gives virulence to the organism. Several variants of the organism may occur: (a) *Smooth*, with and without Vi antigen, but containing O antigen; (b) *Rough*, with and without O, and without Vi antigen. Motile and non-motile variants of each type may also occur. The greatest virulence is associated with the smooth variant containing O and Vi substances. The Vi component is thought to be especially effective in preventing O agglutinins from acting against that antigen because Vi may cover the surface of the organism and so dominate the antigen-antibody reactions, these being essentially surface phenomena.



ever, they have meanwhile continued their activities in the Peyer's patches, multiplying enormously, to the great distress and danger of the patient. Unable to handle the large numbers of invaders, and the retaining mechanisms of the Peyer's patches falling into disrepair, by the end of the first week or ten days of the disease millions of the bacilli escape into the intestinal contents and are removed by defecation. The bacteriologist then turns to the stool examination for diagnosis by laboratory means.

The activities of the organisms in the Peyer's patches may result in perforation of the intestine, in which case the patient dies of peritonitis. As often happens, the invaders, which depend on their host for life and nutriment, perish with him. Thus, the short-sighted parasite destroys its own haven of protection.

*Carriers.*—If a patient survives the attack of fever, he may in a few instances continue to harbor the bacilli in some secluded retreat in the tissues or gallbladder for months or years, yet appear perfectly healthy. These organisms are often excreted regularly or intermittently in the feces and the convalescent becomes a carrier and a menace to the health of others.<sup>15, 16</sup> "Typhoid Mary," a famous typhoid carrier of the last half century, was especially dangerous because, being an excellent cook, she could (and did) get many well paid positions in restaurants, hotels and homes, and thus spread typhoid fever in the food wherever she worked. She was warned and threatened by health authorities and repeatedly jailed. She died a few years ago of natural causes.

After recovery, the blood begins to show diminution of agglutinins, especially of the Vi and O antibodies. If the patient becomes a carrier, his blood often continues to have a demonstrable titer of Vi agglutinins, but these also tend to disappear with complete elimination of the bacteria. Detection of Vi agglutinins in the blood of an apparently healthy person usually indicates that that person is a carrier, since the continued presence of Vi agglutinins in the blood seems to be directly dependent upon the actual presence in the body of typhoid bacilli.<sup>17-22</sup>

**Genus *Shigella* and Bacillary Dysentery.**—A number of the shigellas (also commonly known as dysentery bacilli), especially *S. dysenteriae*, cause intestinal disturbances ranging from very mild, subclinical diarrhea to severe inflammation and ulceration of the large bowel, often with scar formation and stricture of the bowel after recovery (*bacillary dysentery*).<sup>23</sup> In some epidemics of bacillary dysentery, especially those due to *S. dysenteriae*, the fatality rate is high.

*Shigella dysenteriae* gives off a potent exotoxin which is, in part, responsible for the severe symptoms often accompanying infection by this member of the group. In this country *S. dysenteriae* is rarely found in carriers but occurs in some epidemics. Other species, listed below, often cause sporadic and epidemic infections in this country and elsewhere and are commonly found in healthy persons everywhere. Several of these closely allied organisms cause similar conditions. They are much less toxic than *S. dysenteriae*. Among them are *S. ambigua*, *S. paradysenteriae*, *S. Newcastle*, *S. sonnei* and *S. madampensis*. These may be distinguished from the very toxic *S. dysenteriae* by the characters shown in Table VIII. A

TABLE VIII  
SOME CULTURAL REACTIONS OF THE SHIGELLAS

Species	Lac- tose	Dex- trose	Mann- ite	Mal- tose	Su- crose	Indol Pro- duc- tion
<i>Shigella dysenteriae</i> . . .	—	+	—	—	—	—
<i>Shigella ambigua</i> . . . . .	—	+	—	—	—	+
<i>Shigella paradysenter- iae</i> . . . . .	—	+	+	±	±	+
<i>Shigella sonnei</i> . . . . .	Slowly +	+	+	+	+	—
<i>Shigella madampensis</i> . .	Slowly +	+	+	+	+	+
<i>Shigella alkalescens</i> . . .	—	+	+	+	—	+

species which Neter<sup>23</sup> has long pointed out as a probable pathogen. *Sh. alkalescens*, should also be included here.<sup>24</sup>

**Dysentery.**—The means of transmission and prevention of dysentery due to members of the *Shigella* group are similar to these aspects of typhoid fever and salmonellosis, except that animals do not transmit typhoid and dysentery (Fig. 234). Dysentery sometimes occurs in water-borne outbreaks, but milk is a particularly favorable medium for the shigellas, and infants are frequently infected if an adult carrier (which is far from rare) unwittingly contaminates the baby's milk and then leaves it in a warm kitchen to incubate. This is the usual situation in "summer complaint."<sup>25-28</sup>

In severe dysentery, the Peyer's patches in the intestine become



Fig. 234.—Flies abound here. Unscreened privies and dwelling houses are close by in the background. The intermingling of human and bovine dung, contaminating food and milk, is fairly complete. Milk easily becomes contaminated from the feces in the privies via the flies. (Hygienic Laboratory Bulletin 56.)



Fig. 235.—Intestine of a child who died of bacillary dysentery. Ulcerated Peyer's patches are seen as whitish areas all over the lining of the intestine. (After A. Castellano.)

ulcerated as in typhoid fever (Fig. 235), but the organisms do not so often invade the blood stream. Tests for H agglutinins are not made, since the shigellas are nonmotile.

The methods of laboratory diagnosis are similar to those used for salmonellosis except that, because citrates make the plating medium too inhibitory for many dysentery organisms, the formula for desoxycholate citrate medium,\* if used, is modified to exclude these salts.† The selenite medium, so useful for typhoid, is not so successful in isolating dysentery bacilli. "S-S" medium is excellent.<sup>28</sup>

Carriers of dysentery are common. The use of sulfonamid drugs has proved useful in curing them.<sup>29</sup>

**Paracolon Organisms.**—The group of bacteria called paracolon organisms may be regarded as occupying an intermediate position between the *Escherichia-Aerobacter* genera, and the *Eberthella-Salmonella-Shigella* group. The paracolon organisms do not yet have the status of a separate genus, as their relationships are not fully understood. Many workers have referred to them as

**\* Desoxycholate citrate agar:**

Pork infusion . . . . .	1000.0 cc.
Peptone . . . . .	10.0 gm.
Agar . . . . .	20.0 gm.
Lactose . . . . .	10.0 gm.
Sodium citrate ( $2\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 11\text{H}_2\text{O}$ ) . . . . .	25.0 gm.
Sodium desoxycholate . . . . .	5.0 gm.
Lead chloride (optional) . . . . .	3.5 mg. (1 : 300,000)
Ferric ammonium citrate (green scales) . . . . .	2.0 gm.
Neutral red . . . . .	20.0 mg. (1 : 50,000)

† Desoxycholate agar (Leifson): Products of Baltimore Biological Laboratories\* Baltimore, Md.

1. Distilled water (or pork infusion) . . . . .	1000 gm.
2. Peptone . . . . .	10 gm.
3. Agar . . . . .	17 gm.
4. Sodium desoxycholate . . . . .	1 gm.
5. Sodium chloride . . . . .	5 gm.
6. Lactose . . . . .	10 gm.
7. Ferric ammonium citrate (green scales) . . . . .	2 gm.
8. Potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) . . . . .	2 gm.
9. Neutral red (1 percent) . . . . .	3 cc.

Add peptone to water and when in solution, add alkali to an approximate pH of 7.5, boil, and filter if necessary. Add agar and after soaking for at least fifteen minutes, melt by boiling or in autoclave. To the melted agar add the above ingredients in the order given, leaving out neutral red until after titration. Titrate to pH 7.3 if medium is used for isolation of the intestinal pathogens; for milk work titrate to pH 7.5. Add the neutral red from stock solution and heat in steam (100° C.) long enough to kill vegetative cells, fifteen minutes for tubes. Store in dark place.

"aberrant coliforms." They have some of the cultural properties of the coli-aerogenes organisms, and some of the pathogenicity (probably) and antigenic structure, as well as some cultural properties, of the typhoid-paratyphoid-dysentery group. A frequent property of the paracolons is inability to ferment lactose, or a *much delayed* lactose fermentation. When colonies of these organisms are seen on plates of the selective dye media described in this chapter, they are colorless and are often mistaken for organisms of the group of definite nonlactose-fermenting pathogens.

One group of paracolon organisms is related to *Aerobacter*, another to *Escherichia coli*, and another to *E. freundii*, based on I M Vi C relations and antigenic structure. Those related to *aerobacter* closely resemble the members of this genus but ferment lactose slowly or not at all. A similar relationship exists between the *freundii*-like paracolons and *E. freundii*, and so on. As shown by Stuart, Wheeler, Borman, Rustigian and others, the paracolon group is large and culturally and serologically complex. Paracolon organisms are frequently found in food-infection outbreaks and are often mistaken for *Salmonella* unless very complete cultural and antigenic studies are made of them.<sup>30, 31, 32</sup>

**Laboratory Diagnosis of Typhoid Fever, Dysentery, and Related Diseases.—**  
*Blood Cultures.*—In these diseases invasion of the blood stream sometimes occurs. It regularly takes place in the early stages of typhoid fever, less commonly in *Salmonella* and *Shigella* diseases and probably rarely in paracolon infections. Accordingly, in typhoid fever, the first attempt at diagnosis during the first week after onset consists in blood cultures. From 5 to 10 cc. of blood are drawn from a vein and transferred to a flask containing 100 cc. of 2 percent bile-broth. If desired, the blood may be allowed to clot and the serum used to test for H, O and Vi agglutinins, although these appear in greater concentration later (after ten days). The clot will enmesh any bacilli, along with the blood cells, and may be macerated in 100 cc. of infusion broth containing about 2 percent bile. After incubation, selective agar plates are streaked for isolation in pure culture.

*Cultures from Feces and Urine.*—When isolation of *Eberthella typhosa* from the feces is attempted, a bit (1 gm.) of the fresh specimen may be placed in a tube of fluid medium containing sodium selenite, and tightly stoppered. It is incubated for about fourteen hours. The selenite partly inhibits growth of the large numbers of *Escherichia* and other organisms present in feces but permits *E. typhosa* to multiply. This method is less useful for *Salmonella* and is of less value for *Shigella* since the selenite is toxic for the latter organisms. No data are available for paracolons.

After about fourteen hours' incubation of the selenite medium, plates of appropriate selective medium, as already indicated, are streaked and incubated for eighteen to twenty-four hours. These cultures often yield results when plates inoculated with the fresh stool specimen fail to yield the desired organism.

A sample of the fresh stool is also streaked on the surface of an agar medium designed to allow the growth of *E. typhosa*, etc., and to eliminate or retard growth

of other organisms (see section on selective bacteriostasis, page 126). Among many such selective plating media for *E. typhosa* and the salmonella group is desoxycholate citrate agar.\* The colonies of *E. typhosa* and the *Salmonella* group on this medium are gray and translucent, round, and glistening. Colonies of other organisms either do not appear, because of the inhibitory action of the desoxycholate and citrate, or are usually easily distinguished from those of the specific pathogens by their appearance. This is true especially if they be lactose-fermenting organisms, *E. coli*, etc., the colonies of which turn red because of the effect of the acidity of the colony, from fermentation of lactose in the medium, on the neutral red. Another good medium for *E. typhosa* and all related organisms is "S-S" agar.† This acts similarly to the desoxycholate agar, but is less inhibitory.

Suspicious colonies on any of the plates are fished with a needle to tubes of sterile broth or nutrient agar as pure cultures. Once isolated in pure culture from blood culture or from feces by means of selective cultivation as described above, the organisms are subjected to biological studies including determination of their distinctive metabolic properties (see accompanying tables) and their agglutination reactions.

*Serological Diagnosis.*—For the serological study of enteric diseases such as typhoid fever, the method of receptor analysis is most reliable. An increase in O agglutinins in the patient's serum from an initially low titer to a definitely higher titer as the disease progresses is especially significant (see section on immunological methods, page 303).

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\* Prepare same as desoxycholate agar. See footnotes, p. 487.

† Product of the Digestive Ferments Co., Detroit, Michigan.

Beef extract . . . . .	5.0	gm.
Proteose Peptone . . . . .	5.0	gm.
Lactose . . . . .	10.0	gm.
Bile salts No. 3 . . . . .	8.5	gm.
Sodium thiosulfate . . . . .	8.5	gm.
Ferric citrate . . . . .	1.0	gm.
Brilliant green . . . . .	0.33	mgm.
Agar . . . . .	13.5	gm.
Neutral red . . . . .	0.025	gm.
Water . . . . .	1000.0	cc.

Boil to dissolve. Do not autoclave.

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## CHAPTER 30

## THE SPIRAL RIGID BACTERIA. CHOLERA

FOR A LONG TIME after the observations of Leeuwenhoek, spirally twisted or curved bacteria were among the most frequently observed. This was due to the fact that they are often relatively large, often motile, occur plentifully in stagnant pond water or woodland pools, and often grow well in very simple solutions. Among these were the flexible spirochetes and the rigid Spirilleae.

Although some spiral organisms were early recognized as bacterial in nature, there is still some confusion as to the manner in which the Spirilleae should be classified. At present, four genera are recognized: *Vibrio*, *Spirillum*, *Cellvibrio* and *Cellfalcicula*. The names of the last two genera are derived from their ability to digest cellulose. All are rigid, curved or spiral in form, nonspore-forming, gram-negative, and usually motile. It is to be borne in mind that these spiral or curved organisms are distinct from the similarly named Spirochaetaceae (order Spirochaetales). The cells of the Spirilleae are not flexible. Spirochetes are flexible. The Spirilleae are true bacteria, while the spirochetes have a number of characters in common with protozoa. The Spirilleae are saprophytic soil and water organisms in general, although one or two dangerous pathogenic species are found in the genus *Vibrio*.

**Genus *Vibrio*.**—This genus includes rods, always curved or bent in one plane and sometimes slightly curved in a second plane



(Fig. 236). Their length seldom exceeds five microns. They are motile by means of one or more polar flagella and are aerobic. Some of them are facultatively anaerobic.

There are almost two dozen "species" of vibrios, all closely related, many of which greatly resemble each other.<sup>1</sup> Here is an excellent example of the tendency, previously noted, to base species distinctions on relatively slight differences. Here also is an instance of a group of organisms ranging themselves in a sort of graded series as regards metabolic activities. At one end we find *Vibrio percolans*, nonpathogenic, not attacking carbohydrates, not liquefying gelatin or serum, not producing indol, not changing



Fig. 236.—*Vibrio comma* ( $\times 900$ ).

milk, not reducing nitrates—in short, metabolically inert. At the other end we find *Vibrio comma*, causing Asiatic cholera, digesting milk, fermenting numerous carbohydrates (without gas), liquefying gelatin and coagulated serum, forming indol, reducing nitrates and producing hydrogen sulfide. The other species could be roughly graded as to metabolic activity between these two extremes.

The group of vibrios, as a whole, is of little importance except as scavengers living almost entirely in water. *V. comma* resembles others in living for long periods in aquatic habitats, but is a striking exception in being the cause of Asiatic cholera. Vibrios, very closely

related to *V. comma*, and almost indistinguishable from it, are sometimes found in the stools of persons ill with enteritis of more or less severity, but they have an unknown etiological relationship to such conditions. As is true of many *Salmonella* types, these vibrios are distinguished by place names, such as *V. gindhā*, *V. danubicus* and *V. massauah*. A species possibly of greater pathologic importance is called *V. proteus*. These are nearly all serologically different from *V. comma*, and may be distinguished by agglutination reactions and Pfeiffer's test (see below).

One vibrio species which probably does cause a cholera-like disease is known as the *El Tor vibrio*. It was first found among pilgrims to Mecca who were suffering from cholera. El Tor is the name of a town on the western side of the Sinai peninsula. This vibrio is strongly hemolytic. The others are not. The miscellaneous species sometimes cause confusion in attempts to diagnose various enteric diseases bacteriologically, especially in India and the Orient. Some other vibrios, like that discovered by Metchnikoff (*V. metchnikovi*), are pathogenic for lower animals like guinea-pigs and pigeons, which is not true of *V. comma*.

**Pfeiffer's Reaction.**—In this test, the organisms are injected into the peritoneal cavity of an *immune* guinea-pig. The peritoneum is punctured at short intervals thereafter and organisms withdrawn for examination. They are seen to undergo progressive lysis due to the action of the amboceptor and the complement in the guinea-pig's blood serum. The reaction is highly specific. Normal pigs give no such reaction. This test gives a striking illustration of the general principle of specific bacteriolysis. It may be demonstrated in the test tube using immune serum (amboceptor), fresh complement and the specific vibrio. It is also readily demonstrable in relation to many other organisms, notably *Leptospira icterohemorrhagiae* with serum from patients recovered from Weil's disease caused by that organism.

**Isolation of Vibrios.**—There are three physiological characters of several vibrios (and especially *V. comma*) upon which methods for their isolation are based. One is a strong tendency to grow at the surface of nutrient liquids in response to demands for oxygen; second, a preference for, and tolerance of, an alkaline reaction (pH 8 to 9) which retards the growth of many of the bacteria associated with vibrios in fecal material (*e.g.*, *Streptococcus faecalis*, *Escherichia coli*), and third (resulting from the two foregoing properties), very much more rapid growth than most other organ-

isms at the surface of *alkaline egg-peptone*\* solution, so that transfers from the surface film after six to eight hours' incubation generally yield almost pure cultures of *V. comma*.

*Vibrio comma* and its congeners grow well on ordinary laboratory media also. The colonies on agar are small, colorless and translucent. They are markedly proteolytic organisms, liquefying gelatin and digesting casein, as well as being active in the decomposition of carbohydrates like dextrose and sucrose, and alcohols such as mannitol. Lactose is not hydrolyzed by *V. comma*, a property common to nearly all of the pathogenic gram-negative, nonspore-forming intestinal rods such as the typhoid and paratyphoid and most dysentery bacilli.

*The Cholera-red Reaction.*—When concentrated sulfuric acid is added to a peptone solution containing sodium nitrite and indol, a cherry-red compound is formed. This is called the *nitroso-indol reaction*. Any organism which, in simple peptone broth, reduces nitrates to nitrites and *at the same time produces indol*, will give the nitroso-indol reaction when sulfuric acid is added to the culture. *Vibrio comma* exhibits the phenomenon and, as the reaction was at one time thought to be especially characteristic of this organism, it was spoken of as the “cholera-red” reaction. It has since been found that a number of other vibrios have the same property. Not all strains<sup>1</sup> of *V. comma* exhibit the reaction, and some are variable.

**Asiatic Cholera.**—As indicated above, *V. comma* can live in the human intestinal tract. Many healthy carriers of *V. comma* exist in the Orient. Often, especially in epidemic periods, the vibrios seem to invade the lining of the intestinal tract without causing immediate disease. Later, as a result of some unrelated gastrointestinal disturbance, they produce disease which may be mild or fatal.

Like dysentery, cholera is characterized by an intense diarrhea and prostration. The patient suffers chiefly from toxins given off

\* **Alkaline egg-peptone solution** (Goldberger):

“(a) Prepare an alkaline egg solution by first shaking or beating up an egg with an equal volume of water and then adding to this egg-water an equal volume of a 5 percent solution of *anhydrous* sodium carbonate. Steam for three-quarters to one hour. (b) Prepare Dunham's solution: peptone 10 gm., salt 5 gm., water 1000 cc.

“For use mix (a) and (b) in proportion of 1 : 9. Run through paper filter; distribute in 10 cc. quantities in tubes and sterilize by steaming for one and one-half hours, after which they are ready for use. This solution is of a pale straw color. It is opaque, and a slight precipitate settles to the bottom of the tube on standing, but this interferes in no way with its serviceability. It will keep at least a week.”

In emergency, ordinary 1 percent alkaline aqueous solution of peptone may be used, with almost equally good results.

by the vibrios (whether endo- or exotoxins is not certain). Great damage also is done by the dehydration which is a consequence of the diarrhea. The mucosa of the large intestine comes away in flakes and the stool, being thin and watery, is described as "rice-water stool." These flakes of mucosa contain large numbers of microscopically demonstrable cholera vibrios and the organisms may be cultivated from the stools by the use of alkaline-egg-peptone-water medium as already described.

Cholera is confined today to localities where grossly polluted water is used. It is to be found chiefly in seaports and other cities of the Orient. While now rare or absent in the United States, this frightful disease is an ever-present danger in the Orient; the newspapers have recently carried stories of its ravages in China due to conditions resulting from military operations there.

Cholera is a classical example of what results from lack of sanitation, especially with respect to water supplies. A century or less ago, cholera was to be found in practically every large city in the world,<sup>5</sup> particularly in communities where there was much of a transient population or in places which were centers for religious, military or other concentrations of large numbers of people. What were probably the most suitable conditions for the spread of cholera and other enteric diseases existed in India some years ago. Conditions in India have been greatly improved since the following description was written, through the cooperation of educated native rulers and British authorities. It is thought worthwhile to quote this description in some detail because it represents a situation which must have developed in similar manner in many mass migrations of people, such, for example, as the crusades, where cholera and other diseases decimated the armies and the devoted followers. Conditions in areas of China dominated by Japan are similar today. This vivid description, taken from the Report on Cholera to the Secretary of the Treasury, by John M. Woodworth, Supervising Surgeon, U. S. Marine Hospital Service, 1874, runs as follows:

"The festivals and pilgrimages which are so frequent in India have often been charged with regenerating and distributing cholera, as the march of armies is well known to do. . . . Twenty-four high festivals take place every year at Juggernaut, below Calcutta. . . . Day and night, through every month in the year, groups of devotees pour along the great Orissa road, from Calcutta, and for three hundred miles every village has its pilgrim encampment. The parties consist of from twenty to three hun-

dred persons, and at the time of the great festivals these bands follow so closely as to touch each other. . . . They often travel one thousand to one thousand four hundred miles by rail, but generally have to walk from three hundred to six hundred miles, and are always forced up to doing a full day's journey. Many a sickly girl and feeble man dies on the road, and all arrive lame, with their feet bound up in rags, plastered with dirt and blood. . . . Disease and death make havoc of the pilgrims, for they are badly lodged and poorly fed. . . . The natural drainage of the place is checked by sandy ridges, and the city is a very dirty one. Each house is built upon a mud platform about four feet high, in the center of which is a hole which receives the filth of the house-

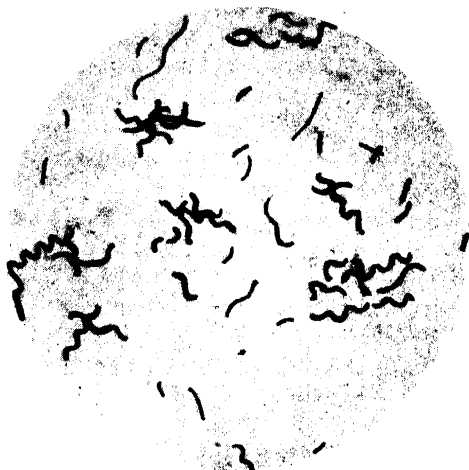


Fig. 237.—*Spirillum rubrum* ( $\times 900$ ).

hold. The wretched inmates eat and sleep around this perennial source of death. . . . Cholera invariably breaks out. The living and dead are huddled together, with a leaky roof above, a foul cess-pool below, and with only just as much space as they can cover lying down."

"As regards the spread of cholera among the villages in India, . . . the history of the outbreaks of cholera is the same in every case. A case of sporadic cholera occurs in a Hindoo village; the patient is placed in the smallest and closest apartment of the house; a large fire is lighted, and as many people, friends and relatives of the sufferer, as the room will hold, assemble and squat around him solely for the purpose of praying, as there is seldom,

if ever, any attempt to administer remedies. The patient during this time is vomiting, defecating, etc., and the fomites of the disease must necessarily be carried off by the visitors to the crowded rooms and huts. After three or four days, five or six new cases occur, and so on daily, until it runs through the whole village or villages to which the persons first infected belonged. Its further progress is limited or circumscribed by the rude system of quarantine maintained by the natives."

In recent years conditions have been improved to the utmost possible extent. Pilgrims are vaccinated against typhoid, cholera and smallpox; medical authorities will not grant passports to diseased persons; ships are inspected and controlled; and sanitation of camps and kitchens is developed to a high degree along modern lines.

**Genus Spirillum.**—This genus has been given relatively little attention by bacteriologists because it contains no species known to be of special interest to the industrialist, or the agriculturalist and only one or two of medical interest. All but the last mentioned members of the genus are harmless saprophytes and scavengers, living in water and putrefying materials.

Morphologically the spirilla are, in general, larger than any of the other Eubacteriales, ranging from 1 to 50 microns in length and from 0.5 to 3 microns in diameter. They are spirally twisted through one or more complete turns (Fig. 237) and are motile by means of polar flagella (Fig. 238). They are gram-negative and produce no spores. They usually grow with difficulty on initial isolation, and their purification by ordinary procedures is not easy. Perhaps this is one reason why they have not been more fully studied. Myers<sup>2</sup> has contrived to cultivate them in pure culture by first preparing infusions of stagnant water, dung or sewage enriched with peptone, meat or fish. After a few days at room temperatures the fluid usually swarms with all sorts of creatures.

Ordinary nutrient agar does not serve well for initial isolation from such cultures. If the fluid from an infusion such as the above

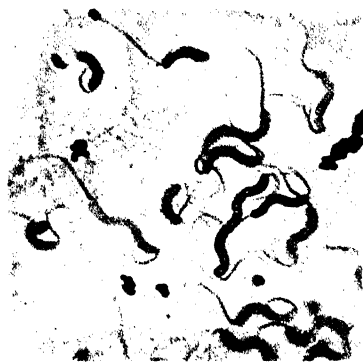


Fig. 238.—Typical spirilla showing flagella.  $\times 1000$ . Note granules. (Myers, Jour. of Bact., Vol. 40.)

be sterilized by passage through a Seitz filter and solidified with 2 percent agar in Petri dishes, growth of colonies can be obtained by inoculation with the unfiltered infusion. However, as the spirilla require much fluid, ordinary streaking out is unsatisfactory. Dilutions of the infusion are made, drawn up into capillary tubes, and blown out in a fine spray over the agar. The plates are incubated for five to seven days at room temperature. If suitably diluted some of the droplets contain single cells of organisms which grow in the drop and form small colonies without distinctive appearance. This is an interesting combination of the dilution methods of Pasteur and Lister with the solid-plate method of Koch.

After initial isolation growth on ordinary medium is relatively easy. The author maintains a strain of *Spirillum* (sp.<sup>2</sup>) on blood-infusion agar or plain infusion agar or broth from year to year.

*Spirillum volutans*, one of the largest species, is of interest because of its metachromatic granules, from which the term *volutin*, in reference to the species, is derived.<sup>3</sup> The motility and form of these spiral organisms is beautifully demonstrated in hanging-drop preparations made from cultures in peptone-tap-water solution and incubated four days at room temperature. Negative staining is an effective means of making the organisms' arrangement visible.

*Spirillum minus* and Rat Bite Fever (Sodoku).—*Spirillum minus*, classified with the spirilla because of its two to four spirals, its rigidity and its polar flagella, is often thought of by medical bacteriologists as a spirochete because (a) it causes in man<sup>4</sup> a disease (rat bite fever or sodoku—from Japanese *so* = rat; *doku* = poison) having several of the fundamental clinical features of a typical spirochetal disease (syphilis); (b) because, like several of the true spirochetes, it has never been successfully cultivated; and (c) because it is rather refractory to ordinary stains. It is gram-negative. *Sp. minus* occurs in the blood of rats and possibly other animals and is transmitted from them to each other and to man by their bites. The organism is rarely found in the normal mouths of rodents but their injured and bleeding gums can contaminate their bites with blood. At the site of the bite of rats in human beings an encrusted ulcer develops, which is analogous to the chancre in syphilis and sometimes, like the latter, contains numerous spirilla (and often streptococci, etc.) demonstrable by darkfield examination and by injection of scrapings into mice. Later, fever of a relapsing type is common and a rash appears, roughly analogous to the rash in secondary syphilis. The Wassermann reaction for

syphilis varies but is said to be positive in about half of some groups of cases. The infection responds rapidly to treatment with drugs like arsphenamine, which are specific for syphilis.

Inoculation of patient's blood or of pus from swollen glands or the chancre-like ulcer into mice and guinea pigs usually results in an infection of the animals, and the organisms can be found in their blood or peritoneal fluid. As laboratory mice and guinea pigs, as well as wild rats, often harbor *Sp. minus* naturally, only animals known to be free of the infection should be used for diagnostic purposes and they should be segregated from other animals so as to avoid infection by bites. The mice may show no signs of illness after injection but the organisms appear in their blood. Blood

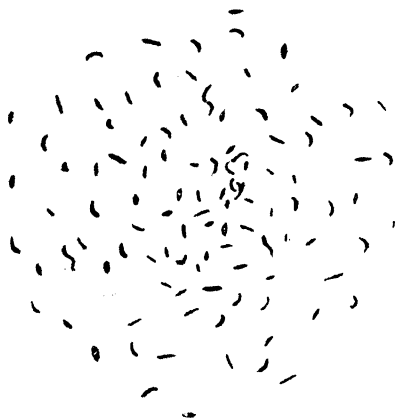


Fig. 239.—*Cellfalcicula viridis* ( $\times 900$ ).

should be examined at short intervals for a month before they are discarded as negative (see also section on *Streptobacillus moniliformis*).

**Genera *Cellvibrio* and *Cellfalcicula*.**—The organisms in these genera are quite similar to each other, the cells of the former genus being usually long, plump, curved rods with rounded ends, those of the latter being relatively short (less than  $2\ \mu$ ), with pointed ends (Fig. 239). All are motile, gram-negative, without spores, aerobic and facultative. They inhabit, especially, soils containing much decaying vegetable matter and are active in cellulose decomposition. In general, they tend toward autotrophism, growing best in the absence of any organic matter except cellulose, although some few species may be induced to grow



on peptone agar, or agar with various carbohydrates. *Cellfalcicula*, however, are specific as to cellulose metabolism.

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## CHAPTER 31

### THE AEROBIC SPORE-FORMING RODS (GENUS *BACILLUS*\*)

ALL SPECIES of this genus are straight rods capable of forming heat-resistant spores and having dimensions ranging around 1.5 microns in diameter and 10 microns in length (Fig. 239a). Most of them are motile. Nearly all species are gram-positive. The members of the genus *Bacillus* are typically strictly aerobic but some can grow a little in the almost complete absence of free oxygen. They are not usually pathogenic, although occasionally some of the common species have been isolated from pathologic conditions in which they appeared to be the causative agent; this is only one of many illustrations of the fact that bacteria which are habitually saprophytic may at times become adapted to a parasitic mode of life.

The genus, as at present classified, comprises some 146 species,† grouped primarily on the basis of their optimum growth temperatures. The first group includes those species growing well at temperatures between 20° and 37° C., the second contains those which are thermophilic (*i.e.*, grow well at about 55° to 65° C.), while a third group contains poorly described species of doubtful validity.

\* In order to avoid confusion of names, the student is urged to re-read the paragraph on page 63 concerning the use of the terms "bacillus" and "bacterium."

† Many of the "species" may be invalid, being variants of one another. However, it is convenient to list them in the keys for purposes of exposition and discussion.

The first, or mesophilic, group is subdivided into smaller groups on the basis of biochemical reactions, size, shape and location of spores, and motility. Because of the very large mass of material, and because all of the species are very similar in many fundamental respects, it seems advisable in this book to do no more than outline the major peculiarities of the group as a whole and point out a few features that appear to be of interest from a practical and philosophical point of view. Because of their large size and widespread habitat, several of the best known species were observed very early and are therefore of historic interest.



Fig. 239a.—Various types of spore-forming rods of the genus *Bacillus*.

**Distribution and Functions of Bacilli.**—The spores of bacilli are almost universally found in soil, dust, and water—everywhere, in fact, on the surface of the earth open to the air. Observations of dust storms which occurred about 1935 in the northwestern part of the United States made it clear that spores of these bacteria and other forms of life are carried thousands of miles by currents of air, while Darwin, as early as 1831, on board the “Beagle,” noted that the dust blown many hundreds of miles out to sea from Africa contained spores, some of them doubtless bacterial (*infusoria*; Ehrenberg).

It is not to be wondered at, therefore, that spore-forming, aerobic bacilli are often a source of embarrassment to the unwary bacteriologist who finds them multiplying unwanted in his cultures. They, with molds and micrococci, are among the most frequent

weeds of the microscopic garden. It requires assiduous care and constant vigilance to exclude them. It was the heat-resistant spores of such organisms that misled Needham, Pouchet and others to support the view that life began spontaneously in the infusions which they *thought* they had sterilized by heating. Even experienced bacteriologists are sometimes embarrassed by their appearance in supposedly sterile material or pure cultures of bacteria. This is usually due to carelessness in the sterilizing room, "short-cuts" in heating processes, etc.

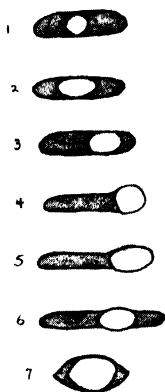


Fig. 240.—Types of sporulation: 1, spherical, central. Cell not swollen; 2, oval, central. Cell not swollen; 3, oval, subterminal or excentric. Cell not swollen; 4, spherical, terminal. ("Drum-stick" sporulation); 5, oval, terminal. Cell swollen; 6, oval, excentric. Cell swollen; 7, oval, central. Cell greatly swollen: "lemon body." (Redrawn from Topley and Wilson.)

If we view evolution as a means of disseminating, preserving and multiplying a given type of living creature, the spore-formers would seem to be a favored group. By means of their spores, they can resist conditions fatal to all non-spore-formers. Further, nearly all of the spore-forming aerobes have a high degree of biochemical activity and have adapted themselves to the utilization of a wide variety of substances as food. A common property is that of hydrolyzing proteins like gelatin, coagulated blood, and dead plant and animal tissues. They also hydrolyze many different carbohydrates, glucosides, alcohols and organic acids. At least one species can utilize carbolic acid (*Bacillus closteroides*). Bacilli are thus seen to be of great importance as scavengers. Some form red or yellow pigments. Some (*B. aerosporus*)

produce acetyl-methyl-carbinol, others hydrogen sulfide, ammonia and carbon dioxide. Also, there are species which prefer to grow at very high temperatures (*i.e.*, are thermophilic), *B. losanitchi* multiplying at temperatures as high as 78° C. These live usually in hot springs such as those at Yellowstone Park.

A peculiarity of the spore-forming, aerobic bacilli is their inability to form spores under *anaerobic* conditions. This is of importance in the study of anaerobes, for the two types may be sepa-

rated by anaerobic incubation followed by heating. The aerobes will not survive.

**Types of Sporulation.**—Much importance has been attached to the location and size of spores as a differential character among

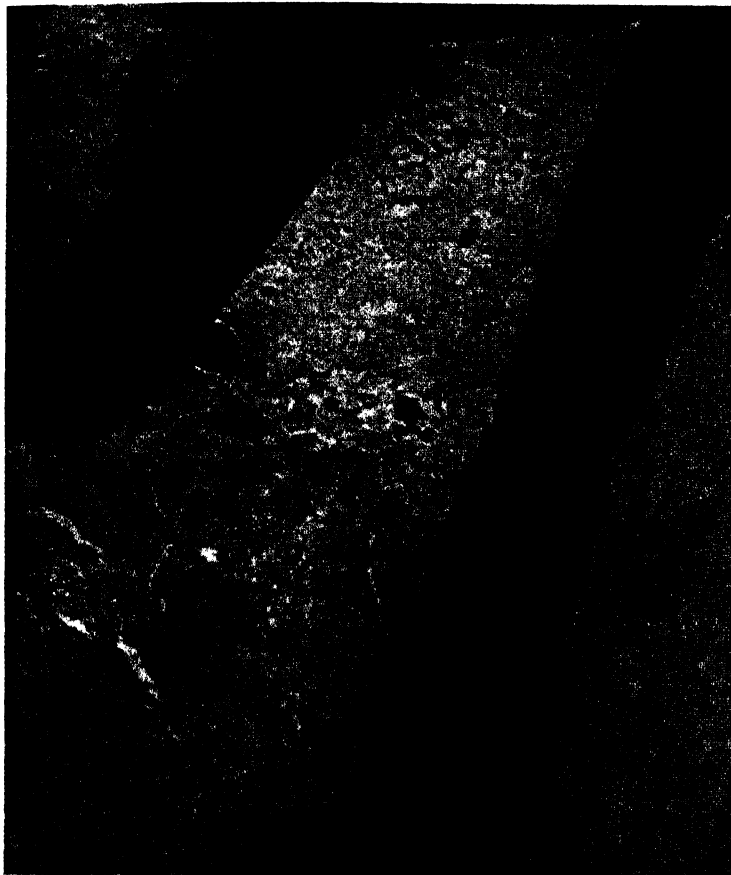


Fig. 241.—Electron microscope picture of *Bacillus anthracis* ( $\times 20,000$ ). Note the capsule and the peculiar plate or thickened cell membrane between adjoining cells. Compare with Figures 242 and 243. (Mudd, Polevitsky, Andersen and Chambers, Jour. of Bact., Vol. 42.)

species of the genus *Bacillus*. Biochemical and other characters are of equal if not greater value in determining species in the group. Spores are said to be *terminal* when they are absolutely

at the end of the rod. Sometimes, when not exactly terminal or definitely central, they are designated as *subterminal* or *excentric* (Fig. 240). In some species the spore is small, having a diameter less than that of the rod. In others the diameter of the spore is much greater than that of the bacillus. Spores may be spherical or oval in shape.

**Species of Special Interest.**—Of this large genus of bacteria, only a few species will be mentioned here. One of these, *Bacillus anthracis*, is very pathogenic. It is not very different from *B. subtilis* as described later in this chapter, but may be distinguished by its lack of motility, its growth in cottony tufts at the bottom of tubes of broth, and its slow liquefaction of gelatin. It also fails to reduce nitrates or to hydrolyze starch, properties which are found in *B. subtilis* and several other members of the genus. Some interesting details of morphology have been revealed by Mudd, et al., by means of the electron microscope. (See Fig. 241.)

**Anthrax.**—This is primarily a disease of farm animals but is transmissible to man.<sup>1</sup> It has been known clinically since antiquity and is probably referred to in the Bible (Exodus ix:9). In man, the organisms most commonly gain entrance from soil or dust to the body through a cut in the skin. They first *localize* at the point of entrance, forming a very rapidly progressive, angry, inflamed pustule (*malignant pustule*), which, when well developed, is covered with a black crust. This pustule teems with anthrax bacilli. It not infrequently heals, but in other cases the bacilli invade the blood stream, multiply enormously, and are spread through all the organs of the body where they tend to form local lesions which serve as further centers for dissemination unless the leukocytes and other defensive mechanisms of the body overcome them. The organism gives off no known toxin but is said to produce death by such great multiplication in the blood as to block the blood vessels. Probably other factors are equally important in causing fatal effects. The generalized disease is highly fatal.

In man or cattle, infection may result from eating infected foodstuffs. Pasteur showed that if sheep were given soft food mixed with anthrax spores only a few died, while if the spores were accompanied by thorny food such as thistles, more sheep died due to the introduction of spores through the injured mucosal surfaces of the mouth. Infection of animals also occurs through abrasions of the skin. Inhalation of dust containing many spores may produce a form of pneumonia due to *B. anthracis*. This is sometimes called "woolsorters' disease" when it occurs in man.

When animals die of anthrax, there exudes from the body openings, for some time after death, a bloody fluid which teems with anthrax bacilli. Although *Bacillus anthracis*, like other aerobic spore-formers, does not form spores under conditions of low oxygen tension such as occur in the blood vessels, the bacilli in these bloody discharges quickly sporulate on exposure to air (Fig. 242). These may remain alive on the ground in pastures for many years, constantly infecting cattle which graze there. Certain pastures in France have thus come to be known as "anthrax pastures." Postmortem examinations of animals suspected of having died of anthrax, should, therefore, never be made in the



Fig. 242.-- *Bacillus anthracis*. Smear from aerobic culture showing spores ( $\times 900$ ).

field. There is too great a danger of infecting many other animals and man since all of the blood and organs of the carcass contain billions of the bacilli which will sporulate as soon as the body is opened. It is sufficient to excise an ear, or make a culture from some of the bloody exudate. The animal should be buried or, better still, burned *on the spot*.

It is thought by some that *Bacillus anthracis* is enabled to withstand the protective mechanisms of the body by means of a thick, mucilaginous capsule which it forms when infecting animal tissues. This is in accord with the general idea that encapsulated organisms are more likely to be aggressive and invasive than nonencapsulated ones (Fig. 243).

**Industrial Hazard of Anthrax.**—It is obvious that persons

working in tanneries and woolen mills are particularly likely to become infected with anthrax, especially by way of the lungs. Anthrax is a menace in the hides and hair industry and every precaution should be, and generally is, taken by the industry and by legislatures to ensure disinfection of hides and wool and proper disposal of infected animals.

A very interesting series of human infections with *Bacillus anthracis* occurred at Camp Merritt, New Jersey, during World War I. A number of soldiers in one organization became infected on the face, all at about the same time. Very typical malignant pustules developed, some of which were treated by cautery. A consignment of shaving brushes, part of which were destined for soldiers overseas, was suspected of causing the infections. Upon cultural examination in the Base Hospital

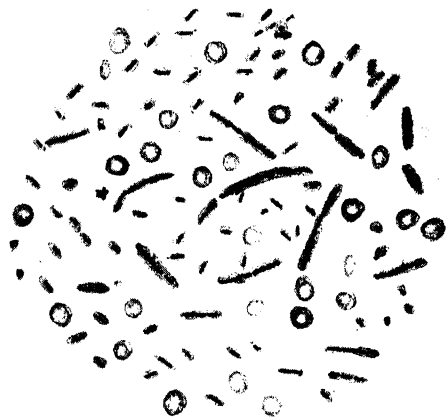


Fig. 243.—*Bacillus anthracis*. Smear from fresh blood showing capsules. The circular objects are erythrocytes. No spores are seen ( $\times 900$ ).

Laboratory each brush in the lot was found to be so full of anthrax spores that a reasonable inference was that the hair or brushes had been purposely infected somewhere en route. Thorough disinfection made the brushes safe and the writer until recently used one that was assigned to him in 1917.

Animals may be artificially immunized by the injection of attenuated organisms, following methods based upon those devised by Pasteur (see section on immunity, page 322) or by the use of serum from immune animals or both. The serum of immune animals seems to have curative value in cases of human anthrax.

**Bacillus Subtilis.**—*B. subtilis* is the type species of the genus and is one of the commonest of aerobic spore-formers. It is found in dusty places everywhere and especially in hay. If hay be soaked

in warm water for a day or two, the water will be found teeming with organisms of many kinds, among which *B. subtilis* will be prominent. Numbers of other species of spore-bearing, aerobic bacilli will also be found. In past decades little or no distinction was made between the various bacteria occurring in hay infusions, since all resembled each other very closely. They were grouped under the general term "hay bacillus." The term should be discarded, but is still used by some almost as a synonym for *B. subtilis*.

*Bacillus subtilis* often forms long chains of bacilli sometimes called "streptobacilli" (Figs. 244 and 245). Since the bacilli are motile, such chains swim with a writhing motion. Individual cells



Fig. 244.—*Bacillus subtilis* ( $\times 900$ ).

or pairs of cells also occur. Due to its avidity for oxygen, *B. subtilis* grows in a scum or pellicle at the surface of fluid media. This is important to remember as it aids in distinguishing between *B. subtilis* and *B. anthracis*. Other characters distinguishing the two are action on nitrates (*B. anthracis* does not reduce them) and action on starch (*B. anthracis* does not hydrolyze it).

Due to its active attack on organic nitrogenous compounds, its cultures smell of ammonia. It grows well on any ordinary organic medium. On slants of potato it grows luxuriantly, with a yellowish or pink color and a warty or vesiculated appearance. The appearance of growth on potato is a means of differentiating between closely related species of spore-bearing, aerobic bacilli, but is subject to much variation





Fig. 245.—Electron Microscope picture of *Bacillus subtilis* ( $\times 12,200$ ). Note the continuous cell wall with thin column of cytoplasm extending from one cell to the other. Note the place in the lower cell where there is a slight indentation and thickening suggestive of a place where cell division may occur. Compare with Figures 241 and 244. (Mudd, Polevitsky, Anderson and Chambers, Jour. of Bact., Vol. 42.)

*Bacillus subtilis* is so easily cultivated, so large and so sturdy, that it has been used as an experimental subject for a great variety of purposes, some of them of commercial value. Bacteriologists

have used it in experiments on the chemical composition of bacteria, in studies of the effect of x-rays and radium, the synthesis of vitamins, resistance to drugs, growth phases, and numerous other problems.<sup>2, 3</sup> It is really a very useful organism.

*Pathogenic Properties of Bacillus subtilis.*—*B. subtilis*, and organisms closely related to it, have been isolated occasionally from pathological processes in human beings. For example, the author obtained pure cultures of an organism indistinguishable from *B. subtilis* from an infection of the lymph glands in the neck of a child. The organism was pathogenic for mice, rabbits and guinea-pigs. Whether the spore-forming rod was actually the cause of the disease in this case it is impossible to say. A few similar instances have been described by Pellegrini,<sup>4</sup> François<sup>5</sup> and others.

**Bacillus cereus, B. mycoides, B. vulgatus, B. mesentericus, B. palustris.**—These organisms are very much like *B. subtilis*. All are "hay bacilli" and all belong to the group of mesophilic species. *B. cereus* forms a spreading, grayish growth on agar, while *B. mycoides* forms very characteristic, moldlike, or nebula-like colonies on agar plates (Fig. 246). On potato it produces a whitish, granular growth which becomes brownish later.

*Bacillus vulgatus* forms a gummy slime and sometimes causes bread to seem "ropy" by growing in it during damp, warm weather, especially if the bread was made with flour containing many spores of this organism. Its growth on potato is thick, pinkish and wrinkled and turns brown.

*Bacillus mesentericus* is a slime-forming species also; its colonies are sticky and mucoid. On potato it forms a moist-looking, wrinkled scum which turns a dirty brown color later. It may be that *B. vulgatus* and *B. mesentericus* are identical organisms.

*Bacillus palustris.*—This species was an obscure soil organism but has come into prominence because, as shown by Sickels and Shaw<sup>6</sup> it produces enzymes which attack the capsules of pneumococci. This enzyme may have therapeutic value.<sup>7</sup> A score or more of very similar species have been found to produce similar enzymes and also antibacterial substances, such as the important

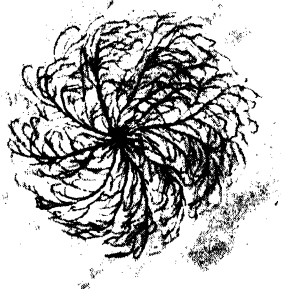


Fig. 246.—Diagram of type of colony produced by *B. mycoides* (a rhizoid colony).

drugs gramicidin, tyrocidin, tyrothricin,<sup>8, 9</sup> etc. These drugs are discussed elsewhere (see section on microbial antagonistic substance, page 140).

**"Milky White" Disease.**—Two other species, called *B. popilliae* and *B. lentimorbus* are now used to combat Japanese beetles. The organisms occur in the "blood" of the larvae and cause the disease commonly known as "milky white" disease. In preparing cultures to combat the beetles, larvae are inoculated and the organisms multiply. The juices are dried and ground and mixed with chalk dust or other powder. This is applied to the soil as a spray or dust.<sup>10</sup> S. R. Dutky developed the process and assigned his patent rights to the government. It is earnestly hoped that the method will help control the beetles!

***Bacillus rotans*<sup>11</sup> and *B. alvei*.<sup>12</sup>**—These species are of especial interest because they produce colonies which move. Instead of merely spreading outward, the whole colonies move in a rotatory manner and actually travel from place to place. The individual bacilli composing the colonies are also motile. These motile colonies are discussed in the chapter on Myxobacteriales (page 379).

**Lactose-fermenting Bacilli.**—Certain species of spore-forming aerobic bacilli are of importance as sources of error in the examination of water for sewage pollution (see page 435). They ferment lactose with gas formation and grow aerobically, and may be mistaken for *E. coli* or *A. aerogenes*.<sup>13</sup>

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## CHAPTER 32

### ANAEROBIOSIS. GENUS CLOSTRIDIUM

**Anaerobiosis.**—Prior to the investigations on “diseases” of beer and wine carried out by Pasteur about 1860, it had been supposed that, in order to live, every creature was obliged to have air. The discovery of oxygen by Priestley in 1774 and the observations of Lavoisier on the nature of combustion were supposed to have provided a solid basis for the conclusion that oxygen is necessary for life. In 1861, however, Pasteur proved that certain micro-organisms could multiply in the absence of air (or free oxygen). He devised the term “anerobiosis” to describe this sort of existence. This was one of the most momentous discoveries in biological science. Subsequent studies in the physiology of cells living in situations devoid of free oxygen revolutionized ideas of cell physiology and metabolism. Pasteur’s observations concerned several species of yeasts and certain spore-bearing bacilli. Since Pasteur’s researches a very considerable number of bacteria capable of living without air have been discovered, including facultative anaerobes and strict anaerobes like *Bacteroides*, *Clostridium*, etc. The chemical mechanism of anaerobic life has already been discussed (see section on metabolism, page 360).

Until recently the systematic study of strictly anaerobic bacteria had not been as extensive as studies of aerobic and facultative organisms largely because of the technical difficulties involved in producing environments free from uncombined oxygen and maintaining them so. Another great stumbling block in the progress of investigation of these organisms in the past has been the isolation of pure cultures. Many anaerobic bacteria are of great medical importance.<sup>11</sup>

**Methods for the Cultivation of Anaerobic Bacteria.**—For many years anaerobic technics have been based on the assumption that free oxygen (air) must be removed from cultures of anaerobic organisms and absolutely excluded from them during the period of growth. This assumption still seems to be correct in a general and fundamental sense. However, knowledge of the relationship between anaerobes and their gaseous environment has advanced to a point where it is possible to cultivate these organisms in ordinary culture vessels freely exposed to air and to eliminate to a great extent the use of the relatively clumsy and elaborate anaerobic apparatuses hitherto thought indispensable to investigations in this field. On the other hand, under some circumstances the apparatuses of the past are still highly useful and widely employed, as well as of historical interest, so that a description of several of them is desirable here.

In spite of the fact that many types of anaerobic device exist, only one fundamental purpose is involved. This is the removal and exclusion of all oxygen from the atmosphere and culture medium. It is brought about by three main classes of procedure.

(1) *Chemical Anaerobiosis.*—(a) Cultures may be enclosed in an airtight vessel with a freshly made mixture of potassium hydroxide and pyrogallol. The combination of these two substances absorbs large amounts of oxygen and leaves only the inert gas, nitrogen, and a partial vacuum (Fig. 247). Spray<sup>1</sup> has adapted this principle to use with Petri dishes. The chemicals are placed in shallow separate compartments at the bottom of a specially devised vessel and the agar plate, after inoculation, is inverted over the top. The joint is sealed with paraffin or plastic. After the seal is complete, the vessel is tilted slightly to mix the pyrogallol and alkali solutions (Fig. 248).

(b) Similarly, if the potassium hydroxide and pyrogallol be replaced by sticks of phosphorus (in a *metal container!*), these will combine with all free oxygen to form phosphorus pentoxide and leave, also, only nitrogen and a partial vacuum.

(c) The combustion of small amounts of alcohol or the burning of a small candle in a closed jar will also use up much of the free oxygen, replacing the air with a mixture of nitrogen and carbon dioxide. Combustion ceases when the carbon dioxide content approximates 10 percent. These methods result in only partially anaerobic conditions and are more widely used to increase the carbon dioxide content of the atmosphere, a condition favorable to many organisms, both aerobic and facultative (see cultivation of gonococci and *Brucella*, pages 611 and 628).

(d) An effective means of achieving an absolutely anaerobic condition in cultures, using the apparatus devised by McIntosh and Fildes,<sup>2</sup> is to allow a fine stream of hydrogen to enter a closed vessel, impinging, as it enters, on a small mass of some catalytic agent which causes it to combine with the free oxygen, forming water. The catalyst generally used is "platinized asbestos" (finely divided platinum



Fig. 247.—Buchner's anaerobic tube. The fluid at the bottom of the large tube consists of pyrogallie acid dissolved in 10 percent NaOH solution. The small tube contains the culture. By Wilson's method the tubes are charged with pieces of caustic potash covered with pyrogallie acid. A minimum of water is added just before closing the outer tube. (Park and Williams, "Pathogenic Microorganisms," Lea and Febiger, publishers.)

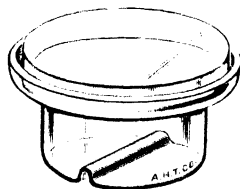


Fig. 248.—Anaerobic apparatus devised by Spray.

deposited on asbestos fibers). This is effective only when heated. In some types of modern apparatus, the necessary heat is applied to the catalyst by means of an electric current passing through a

resistance wire surrounding the mass of catalyst. Calcium chloride or some other drying agent is enclosed in the vessel to absorb the water that is formed. In the modification devised by Brewer,<sup>3</sup> danger of explosion due to sparks is eliminated by enclosing the heating element in a gas-tight tube inside the catalytic mass (Fig. 249).

(2) *Replacement Methods*.—Another method of removing oxygen from the atmosphere of closed “anaerobic jars” is simply to flush

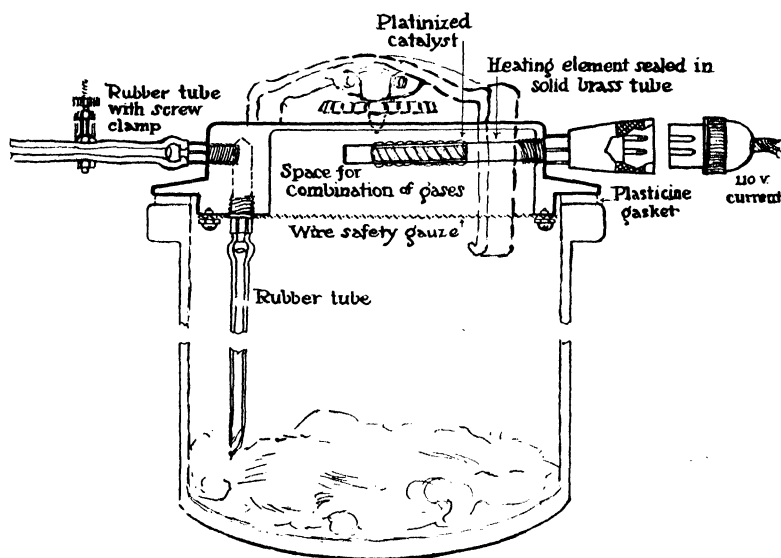


Fig. 249.—Brewer Anaerobe Jar. Note the tube for admission of hydrogen, the enclosed heating element and the platinized asbestos wrapped around it. The upper portion of the jar, where the heating element is located, is separated from the rest of the space by a wire gauge screen, a safeguard based on the principle of the Davy miner's lamp. (Brewer, Jour. Lab. and Clin. Med., Vol. 24. C. V. Mosby Co., publishers.)

out all the air with a stream of some inert gas like hydrogen or nitrogen. This leaves an atmosphere of almost pure hydrogen or nitrogen and no vacuum.

(3) *Oxygen Exclusion Methods*.—(a) A simple means of excluding oxygen from single culture tubes is to boil or autoclave them just before use, cooling rapidly in ice water (so that oxygen is not reabsorbed) before inoculation. The heat drives off the oxygen. The tubes, if of broth, are then sealed by putting a layer of oil,

petroleum jelly or melted agar, several centimeters thick, on the surface. If the organism is a gas-former, the gas may blow the petroleum jelly or agar plug out of the tube. This method is "messy" and not highly effective if very strict anaerobiosis is desired. Withdrawal of material from such sealed tubes is awkward.

(b) We are indebted to Brewer<sup>4</sup> for one of the simplest, yet most effective, means of obtaining pure cultures of strict anaerobes. This depends on chemical absorption of oxygen from air trapped in a very thin layer over the surface of special agar medium in a Petri dish. The cover of the dish is modified by having most of the central portion depressed so that it comes very close to the agar in the bottom part of the dish. A deeper annular depression near the rim actually comes into contact with the agar so that the central part of the agar surface is entirely sealed in, with a layer of air above it only about 1 mm. in thickness (see Fig. 250). The oxygen in this air is absorbed by sodium thioglycollate or some

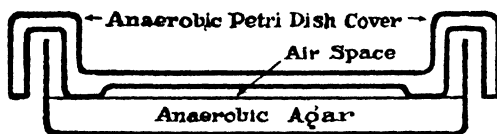


Fig. 250.—Cross section showing Brewer anaerobic Petri dish cover in use

other similar compound having an affinity for oxygen incorporated in the agar.

The agar should be distributed in about 40 cc. amounts in 15-mm. Petri dishes. The depth of agar in the dish should be such that the annular depression in the cover rests on the surface of the agar. The agar plates should be allowed to dry for 24 to 48 hours in the incubator (or use porcelain tops) before inoculation, especially for spreading organisms like *Cl. tetani*. Any good infusion agar or blood agar base may be used. The thioglycollate is used in 0.2 percent concentration. Colonies of *Cl. tetani* develop readily under such conditions.

(c) Deep tubes of dextrose infusion agar are also used to cultivate anaerobes and isolate them in pure culture. Infusion agar in tubes 8 to 10 cm. in depth is melted and cooled to about 50° C. The inoculum is put in and mixed thoroughly. The agar is then made to solidify rapidly in cold water and is incubated. Strict anaerobes will grow only in the depths and will not appear at all within a centimeter or more of the surface. Less strict anaerobes will grow



in the depths and will also grow somewhat nearer to the surface, while facultative anaerobes will grow on the surface as well as in the depths. Organisms having a narrow zone of tolerance to both oxygen and strict anaerobiosis (*microaerophils*) may grow in a narrow zone some distance below the surface (Fig. 251).

In order to obtain pure cultures from such preparations, it is necessary to heat the tube in a hot flame, care being taken to melt only the layer of agar in immediate contact with the glass, and then to place the flamed mouth of the tube directly in a sterile covered dish. A flame is immediately played full upon the bottom

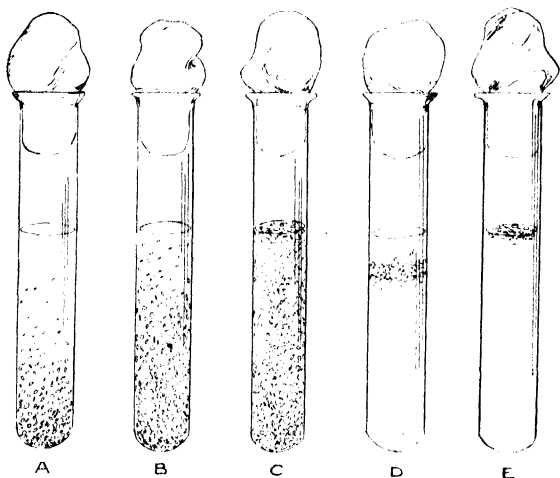


Fig. 251.—Deep tubes of agar inoculated with bacteria of various oxygen relationships. *A* Fairly strict anaerobe, like *Cl. botulinum*; *B* less strict anaerobe, like *Cl. welchii*; *C* facultative aerobe-anaerobe, like *Esch. coli*; *D* micro-aerophilic organism like *Br. abortus*; *E* strict aerobe, like *Pseudomonas fluorescens*.

of the tube. Steam will force the column of agar out into the dish where it may be sliced with a sterile wire or knife wherever desired colonies are located. Transfers may be made from the slice to cooked meat, etc.

The method is awkward. Tung<sup>5</sup> has devised a modification, based on the procedure of Burri, which facilitates removal of the agar column. A vertical glass cylinder about 15 cm. long and 1.8 cm. in diameter is closed at the bottom end with a rubber stopper into which is thrust a hook-shaped glass rod. The agar is put into the tube, which is then plugged at the open top end with cotton, and autoclaved in an upright position. Inoculation is made as

with an ordinary shake tube. To remove the agar column one gently withdraws the rubber stopper. The agar follows, drawn by the glass hook (see Fig. 252).\*

**Cultivation of Anaerobes in Media Freely Exposed to Air.**—Pure cultures of the strictest anaerobes may be obtained in ordinary media with no precautions as to exclusion of air, provided some substance be added to the media to combine with oxygen as fast as it is absorbed by the fluid of the culture.

For example, anaerobiosis in tubes of broth is satisfactory if the medium contains bits of chopped tissue, *e.g.*, Holman's "cooked meat medium"† or some modification of it. The tissue acts as a reducing agent. The meat also serves as pabulum for the bacteria. Most anaerobic bacteria grow well in cooked meat medium.

As shown by Brewer,<sup>7</sup> the addition of sodium thioglycollate (0.1%) or sodium formaldehyde sulfoxalate (0.1%), or both, to dextrose broth or similar fluids adapts them to anaerobic requirements. The addition of 0.1 percent agar creates a very slight viscosity which reduces aeration of the solution by convection currents. T'ung<sup>8</sup> has found that the use of 0.2 percent cysteine hydrochloride is as effective, under most conditions, as the reagents mentioned above. In general milk, infusion broth and infusion agar with blood, treated with the reducing reagents, are good culture media for

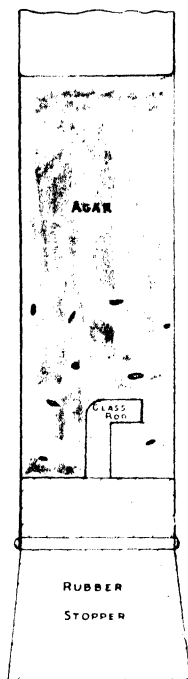


Fig. 252.—T'ung's method of preparing tubes for deep agar cultivation of anaerobes.

\* Courtesy of Ts'un T'ung, Johns Hopkins School of Hygiene and Public Health. From unpublished data.

† **Cooked meat medium:** 250 gm. of fat- and fiber-free beef heart tissue are ground several times through a "fine" meat chopper. Sufficient distilled water is added to about double the volume of the chopped meat and the mixture is brought to a boil. The meat may be passed again through the chopper, replacing it afterward in the water in which it was heated.

Add 0.5 percent peptone, 0.5 percent sodium chloride and 0.1 percent dextrose. Distribute in tubes so that they contain a column 8 to 10 cm. deep consisting of about 4 to 5 cm. of the chopped meat. They may be sterilized by autoclaving.

This medium is usually somewhat acid and may be adjusted in the usual manner but, due to the buffering action of the meat, will require several readjustments after being boiled.

anaerobic organisms, since these organisms require media rich in organic matter and having a *pH* of about 7.2. Chopped brain, fish or other tissues are also often used. The addition of dextrose provides a readily available source of energy which promotes the growth of nearly all anaerobes.

**Purification of Anaerobe Cultures.**—Under natural conditions anaerobic bacteria often occur in mixtures with other species. The separation of species of spore-bearing anaerobes or of nonspore-forming anaerobic species from each other is often difficult but may be accomplished by means of the T'ung tube or Brewer or Spray plate.

*Separation of Aerobic and Anaerobic Spore-formers.*—Cultures of spore-forming bacteria sometimes become contaminated with nonspore-forming bacteria. The culture may be freed from the latter by heating. However, cultures of anaerobic spore-formers may also become contaminated with spore-forming aerobes. Separation may then be difficult. Under anaerobic conditions, anaerobes will form spores that are heat-resistant, while, as previously stated, aerobic spore-formers do not produce spores in the absence of free oxygen. By cultivating the mixture under strictly anaerobic conditions, sporulation of the aerobic forms is inhibited. They may then be killed by heat.<sup>9</sup> McClung has described a very useful method for the isolation and purification of clostridia,<sup>10</sup> which will prove of value to students of the genus.

**Genus Clostridium.**—The bacteria belonging to this genus are all anaerobic, gram-positive, spore-bearing rods. The group includes the organisms producing tetanus (lockjaw), gas gangrene, and botulism (food poisoning), but a majority of the clostridia are harmless and helpful saprophytes. Many of them produce industrial fermentations of great value. Nearly all are motile, and all occur widely distributed in the soil. Some of them also live in the intestinal tract of man and animals. They are metabolically active and versatile.

Many of the pathogenic anaerobic spore-forming rods seem to be endowed with the powers of fermentation and proteolysis in a compensatory manner. That is, those which are highly proteolytic are less actively fermentative and vice versa. There are, however, as in all biological groupings, no absolute lines of demarcation. Table IX, adapted from Brown,<sup>12</sup> shows the chief biochemical properties of some of the common anaerobic spore-formers (clostridia) and gives their names.

In this table the organisms are listed vertically in the order of chemical complexity of carbohydrates which they can attack. They are arranged horizontally according to the chemical complexity of protein which they hydrolyze. Thus, *Clostridium chauvei* (the cause of a gangrene-like disease of cattle, called "blackleg") attacks all of the carbohydrates listed but can hydrolyze only the incomplete protein, gelatin. *Cl. chauvei*, *Cl. perfringens*, *Cl. septicum*, and any other organisms having similar properties, are said to be predominantly "saccharolytic" organisms, while those like

TABLE IX

SOME METABOLIC PROPERTIES OF COMMON ANAEROBIC, SPORE-BEARING RODS\*

Carbohydrates Attacked	Organisms		
Starch, saccharose, lactose and dextrose	Cl. tertium† Cl. chauvei Cl. butyricum‡ Cl. fallax†	Cl. perfringens	Cl. putrificum
Dextrose, saccharose and lactose			
Dextrose, and saccharose or lactose	Cl. septicum and Cl. novyi		
Dextrose only			Cl. botulinum
None	Cl. tetani		Cl. histolyticum
Proteins attacked	Gelatin only	Casein and gelatin	Casein, gelatin, serum, egg, fibrin

\* For explanation of the arrangement see text.

† Does not attack gelatin.

‡ Not a pathogenic species. Closely related industrial species may be included here.

*Cl. tetani* and *Cl. histolyticum* are predominantly proteolytic. Other clostridia are intermediate in these respects.

From a purely taxonomic standpoint, the genus may conveniently be divided into main groups on the basis of size and position of spores, and into subgroups on the basis of biochemical powers. From a practical standpoint we may consider the genus as comprising a pathogenic group, a group of highly fermentative saprophytes of great importance in industry, and a large group of saprophytes of lesser importance. A group which fixes nitrogen

in the soil might also be differentiated but it overlaps with the industrial group. Only a few representative or important species of each group will be discussed in detail.

**Clostridium butyricum.**—Among the earliest to be studied was the organism now used as the type species of the genus *Clostridium*. It is called *Cl. butyricum* (Fig. 253) and represents the group of industrially important clostridia. *Cl. butyricum* has a long bacteriological history. A large number of identical or very closely related types have been studied at various times and given similar names. There are about twenty synonyms for it. These all emphasize its power of attacking carbohydrates, notably starch, with the production of *butyl alcohol* and *butyric acid*. There are numer-



Fig. 253.—*Clostridium butyricum* ( $\times 900$ ).

ous very closely related, possibly identical varieties which have various species names. Their differentiation is difficult. In general, they are large, actively motile, gram-positive rods. Some of them are of great importance in the manufacture of butyl alcohol which is of value in many industrial processes. Organisms of this group of butyl alcohol and butyric acid formers are often spoken of under the general term of “butyric-acid bacteria” or “butyl-alcohol bacteria.” Some varieties, *e.g.*, one called *Cl. acetobutylicum* and others called *Cl. butylicum* and *Cl. pastorianum*, also produce *amyl*, *ethyl*, and *propyl* alcohols and *acetic*, *formic*, and *lactic* acids, *acetone*, carbon dioxide and hydrogen. The products of fermen-

tation depend on the variety of *Clostridium* used and the conditions of the fermentation, i.e., nutrient, pH, temperature, etc. *Cl. acetobutylicum* produces large amounts of acetone and butanol from starchy substrates (see section on Industrial Applications of Bacteriology, page 590). The habit of some varieties or species of the organisms to synthesize and store up glycogen, starch (and possibly other substances) in the form of granules within the cell gave rise to the older generic name *Granulobacter*. The synonyms *Granulobacter saccharobutyricum* and *Granulobacter butyricum* express outstanding properties of these bacteria.

Another important species is *Clostridium felsineum* (Fig. 254). This is closely related to *Cl. butyricum* but is proteolytic. It is



Fig. 254.—*Clostridium felsineum* ( $\times 900$ ).

highly fermentative and has the power of digesting the pectin or cement-like gum of plant tissues so that it is useful in the preparation of flax, facilitating separation of the bast fibers from the rest of the plant tissue (see retting of flax, page 595).

**Anaerobic Nitrogen Fixation.**—An interesting property of some of these butyl alcohol organisms (notably *Cl. butyricum*) is the power to fix atmospheric nitrogen. (See nitrogen cycle, page 413.) They grow well in poorly aerated soils, where they are active in hydrolyzing cellulose and fermenting the products of this decomposition (Chapter 25). They also grow in good agricultural soils.

**Pathogenic Clostridia.**—Although highly dangerous pathogenic organisms, *Clostridium botulinum*, *Cl. perfringens* (long

known as *Cl. welchii*) and *Cl. tetani* are not parasites but strict saprophytes. They grow only on dead matter and cannot invade live tissue. Excepting *Cl. botulinum*, they are all commonly found in the soil and fecal material, especially in horse manure, and the spores, consequently, are widespread in street dirt and manured lands. *Cl. botulinum* is also a soil organism but is not known to be common in feces. Morphologically and culturally they are much like the butyric acid organisms, but tend rather less toward fermentation and more toward proteolysis, although no sharp line of demarcation between the two groups may be drawn.

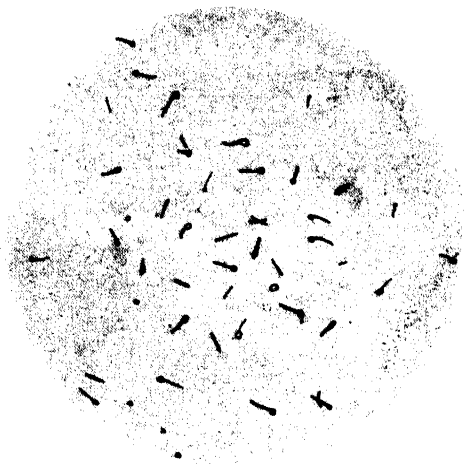


Fig. 255.—*Clostridium tetani* ( $\times 900$ ). Note the terminal spores.

**Clostridium tetani and "Lockjaw."**—*Clostridium tetani* is one of the strictest anaerobes (most sensitive to air). It is cultivable on blood-infusion agar plates or slants only in an atmosphere of inert gas like hydrogen or nitrogen. It grows well in Brewer plates and in broth containing 0.2 percent cysteine hydrochloride, as well as in cooked meat medium. It ferments no carbohydrates, nor is it very active in attacking proteins. Morphologically, the organism is usually a slender rod ( $0.5\mu$  by 4 to  $8\mu$ ). It bears a spherical spore at the very tip end (terminal) of the rod. The round, terminal spore gives to the organism what has been called a "drumstick" appearance (Fig. 255). It is thus very different in appearance from *Cl. perfringens*, *Cl. sporogenes*, etc., which produce oval spores located centrally or excentrically.

Like *Corynebacterium diphtheriae*, *Staphylococcus aureus*, and streptococci capable of causing scarlet fever, *Cl. tetani* gives off a very potent exotoxin. Tetanus toxin is particularly active in the motor nerve centers, irritating them so that the muscles connected with them are thrown into a state of violent and continuous contraction (*tetanic convulsion* or *tetanus*). Once the toxin has combined with and affected the nerves, antitoxin is almost useless.

The organism itself *cannot invade the body*, but grows as a saprophyte on the dead tissue in the wound liberating its deadly toxin. Being a very strict anaerobe, it is able to grow only in deep wounds or those from which air is excluded.

Tetanus toxin, like the toxins of *C. diphtheriae*, *Cl. novyi* and *Cl. botulinum* is one of the most potent poisons known. For example, it requires only about 0.00025 gm. of tetanus toxin to kill a man while it requires twenty times as much cobra venom and about one hundred and fifty times as much strychnine to do the same. However, if tetanus antitoxin be given a wounded patient (victim of street accident, shell-wounded soldier, etc.) very shortly after the wound has been made, any toxin formed in the wound is neutralized before it does any damage. For this reason it is customary in most hospital accident wards, military field dressing stations and the like, to treat all wounded patients routinely with serum containing tetanus antitoxin.

Within the last few years an alum-precipitated toxoid in all respects analogous to diphtheria toxoid (see page 311) has been found useful in producing a lasting, active immunity to tetanus. It is especially useful for military personnel. The protection depends particularly on the value of a primary stimulus as a "conditioner" which enables the body cells to respond very quickly with the production of antitoxin when tetanus toxin gains entrance to the body as the result of a wound. It has been shown that the toxin, *in persons previously actively immunized*, stimulates a rapid rise in tetanus antitoxin titer in the blood. A dose of toxoid in actively immunized persons accomplishes the same thing and is often used as a prophylaxis in dealing with any fresh wound unless tetanus is imminent, when passive prophylactic antitoxin may be used. The same principles apply in diphtheria (see page 312). Combined diphtheria and tetanus immunization has been found entirely practicable.<sup>13, 14, 15</sup>

**Clostridium perfringens** (*The "Gas Bacillus"*).—*Cl. perfringens* is a rather short, thick rod with rounded ends. It usually grows singly, never in long chains or filaments. It forms oval central or



subterminal spores (Fig. 256). With four or five relatively unimportant exceptions, it is *the only nonmotile species in the genus*. It may be pointed out here that, in attempting to determine motility of anaerobic bacteria, care must be taken not to expose the hanging drop to the air for more than a few seconds as motility is destroyed by access of free oxygen. It is a typical anaerobe, although not so intolerant of free oxygen as some other members of the group and is a constant inhabitant of the soil and of the intestine.

Its constant presence in feces has led at times to its consideration as an indicator of human fecal pollution when found in water. There are, however, serious objections to this. For example, it

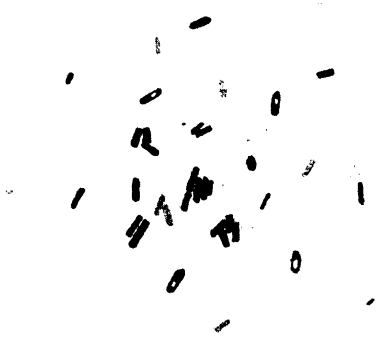


Fig. 256.—*Clostridium perfringens*. Cultivated in cooked meat medium ( $\times 900$ ).

occurs also in soil and in animals and outlasts for years, by means of its spores, any danger of infection by organisms of the typhoid group. It gives falsely positive presumptive tests in bacteriological water examinations (see p. 434) due to its ability to form gas from lactose under the partially anaerobic conditions in the depths of the fermentation tubes used for bacteriological water analysis.

**"Stormy Fermentation."**—Metabolically, it is quite active, liquefying gelatin and fermenting dextrose, saccharose, lactose, starch and other carbohydrates, with the formation of much hydrogen. One of its most characteristic cultural reactions is its power to produce a condition in milk called "stormy fermentation." If a column of milk 10 cm. deep in a culture tube be inoculated

with *Clostridium perfringens*, the lactose is quickly fermented with the formation of sufficient acid to coagulate the casein. Gas is then formed in large amounts and the clot of casein is rent asunder, giving the appearance of a very turbulent or "stormy" reaction (Fig. 257). Any organism producing sufficient acid to form a solid clot, followed by abundant gas production, could give the same reaction. There are, however, only a few such organisms.



Fig. 257.—Tube of milk inoculated with *Clostridium perfringens*, showing "stormy fermentation." (N. MacL. Harris prep.)

***Clostridium perfringens* and Gas Gangrene Organisms.**—*Cl. perfringens*, being common in the soil, always accompanies *Cl. tetani* in wounds and, like the latter organism growing saprophytically only on dead tissue, gives off a toxin which is guarded against in the same manner as tetanus toxin, *i.e.*, by prophylactic injections of antitoxic serum into wounded persons.

In dirty wounds, in addition to *Clostridium perfringens* and *Cl. tetani*, there are nearly always present several other species of

saprophytic clostridia of the soil such as *Cl. septicum* (which invades the blood), *Cl. novyi*, *Cl. histolyticum*, etc. Some of these are able to digest dead tissue rapidly.

*Gas Gangrene*.—The combination of *Cl. novyi* or *Cl. perfringens*, the gas-formers and the other organisms mentioned, in dirty wounds such as crushed members, shell wounds, nail punctures, etc., where there is much dead tissue, if unchecked, produces a rapidly fatal condition known as *gas gangrene*. Many of the organisms produce hemolysins. *Cl. perfringens* and *Cl. novyi*, growing in the dead tissues, produce large amounts of gas. The gas bubbles in the tissues expand and press upon the surrounding live tissues, shutting off their blood supply. The tissues, deprived of their blood supply, die. The highly proteolytic *Cl. histolyticum* and its allies then actively digest this dead, gangrenous tissue and the surrounding parts are thus further invaded by the gas gangrene group which extends the process very rapidly. The toxin given off by *Cl. perfringens* aids in weakening the patient and, without treatment or amputation, death ensues very rapidly.

Such wounds are best opened and cleansed of dirt and dead tissue and exposed to air and disinfectant solutions. During the present war soldiers are equipped with packets of *sterile sulfonamids* for treatment of wounds. Treatment with these drugs prevents gangrene to some extent and is also of great importance in preventing sepsis by other organisms, notably hemolytic streptococci. Potent antitoxins can be obtained to aid in treatment of human gas gangrene. They are principally anti-perfringens and anti-novyi antitoxins.

*Clostridium septicum* (*Vibrion septique*).—This is a longer, more slender organism than *Cl. perfringens* and often grows in long filaments. It produces oval, central spores. It has well marked fermentative (saccharolytic) powers, but it also attacks proteins having a higher degree of chemical complexity than gelatin. It is the cause of a fatal disease (malignant edema) in man and animals, similar to gangrene but without gas formation. There is accumulation of much fluid (edema), and moderate toxemia. The organism often produces *septicemia* (pathogenic bacteria growing in the blood; "blood poisoning"). It is also associated with *Cl. perfringens* and numerous other soil anaerobes in *gas gangrene*. It is of historical interest because it was the first pathogenic anaerobe to be described (Pasteur, 1877).

*Clostridium botulinum*.—This is one of three important organisms (*Staphylococcus*, *Salmonella*, *C. botulinum*) causing food

poisoning. *Cl. botulinum* is a strict anaerobe and forms large, oval spores in a subterminal position, often giving the sporulating rod a shape that is said to resemble a snowshoe (Fig. 258). Like the other clostridia, it is widely distributed in the soil.

It is quite proteolytic and more or less saccharolytic as well. It derives its name from the Latin word for sausage (*botulus*). It was given its name because it was first found in sausages which were the cause of fatal food poisoning. The interior of a sausage obviously presents an ideal place for the growth of anaerobes.

*Clostridium botulinum* is a dangerous pathogen, yet it never grows extensively in or on the human body.



Fig. 258.—*Clostridium botulinum* ( $\times 900$ ).

*Botulism*.—In meat sausage, especially the softer, moist sausages, many different strict and facultative anaerobes find ideal conditions for growth. So also, inside of cans of meat, vegetables and fish, strict and facultative anaerobes may find good pabulum and good anaerobiosis. The bacteria get into the containers when dirty foods are used, having soil or manure on them, visible or not seen. Sometimes the growth is not sufficient to spoil the food and it may be eaten.

Of all the anaerobic and facultative bacteria that get into canned goods and sausages, *Clostridium botulinum* is by far the most dangerous. Like *Cl. perfringens* and *Cl. tetani*, it gives off a very potent exotoxin, which is excreted into whatever material encompasses

it as it is growing. Unlike the toxins of *Cl. perfringens* and *Cl. tetani*, however, the toxin of *Cl. botulinum* remains poisonous when swallowed, being apparently unaffected by the acidity or pepsin of the gastric juice as are most other bacteria and their toxins. Botulinus toxin is absorbed directly from the stomach and intestines. It affects the nerve-muscle complex, producing a flaccid paralysis, particularly of the face and throat. As in tetanus, after symptoms appear antitoxin is of little avail therapeutically and, as no one knowingly eats food containing botulinus toxin, antitoxic prophylaxis is not used. Botulinus toxin may be of three serological types, A, B or C. Type C, and some others, are not common in the United States. There is a specific antitoxin for each type. The toxin is highly pathogenic for mice and other animals as well as man.

Botulism has a high rate. Many cases of botulism (and also *Salmonella* and *Staphylococcus* intoxication) in the past were called by the common, incorrect name of "ptomaine poisoning" (see *ptomaines*, p. 356).

*Botulism and Canned Foods.*—It appears, then, that we might eat moderately spoiled food frequently with no ill effects and even without knowing it, until we happened across a can or sausage in which *Clostridium botulinum* had grown, forming its deadly exotoxin. Then serious illness or death would result.<sup>16</sup> One seldom hears of food poisoning or botulism nowadays. The reason is that commercial canning has superseded home canning to a great extent and that the canning and preserved food industry as a whole, stimulated by accidents in the past, has employed some of the most skilled bacteriologists, chemists and technologists in the world to arrange the food selection, sealing and sterilizing, so that botulism from commercially canned foods is almost unknown in this country today. Due to shortages of food and the advent of millions of "victory gardens," it is to be anticipated that much more home canning will be done than heretofore. It is not unlikely that the frequency of botulism may increase due to improper methods of processing in the home.<sup>17</sup> Home canned foods are dangerous because often the cleaning, sterilizing, preserving or sealing is not properly done. One may easily guard against botulism in any case, however, by boiling all canned foods for twenty minutes after opening them. The toxin of *Cl. botulinum* is destroyed by this amount of heating.

Acid foods like tomatoes, pickles, most acid fruits, etc., seldom cause botulism because *Clostridium botulinum* can grow well only

in slightly alkaline media. Jams, conserves, jellies and sugar syrups likewise do not support good growth of *Cl. botulinum* because of the high osmotic pressure of sugar solutions.

*Precautions in Home Canning.*—In home canning, much can be done toward obtaining safer, more palatable and better looking foods if a few simple precautions are taken:

1. Use only sound foods, fresh as possible, well washed and clean. They should be boiled in a covered vessel, with just enough fluid (water or juice) to cover them. This should continue for at least fifteen minutes, or until they are heated throughout. This will kill many spores.

2. With a clean ladle, transfer the hot food into clean (boiled) preserving vessels and cover as directed by the manufacturer of the container. Do not pack too tightly. Fluid must circulate freely.

3. Place the vessels in the sterilizer. This should preferably be a pressure cooker. If fractional sterilization (tyndallization) is to be used, any covered vessel, such as a wash boiler, may be used. The water in the sterilizer should be boiling.

4. Manufacturers' directions generally accompany pressure cookers. If you have no directions, then, for general purposes, proceed as follows:—

- (a) *Be sure the safety valve, air vent, and pressure gauge are in working order or you may have a fatal explosion.*
- (b) Open the air vent wide. Have about 1 inch of water in the bottom of the cooker.
- (c) Clamp on the cover tightly in the correct position (see arrow on cooker and lid).
- (d) Leave the air vent open until *pure steam* issues with a *loud hissing noise*. Allow seven minutes after boiling begins.
- (e) Close the air vent and watch the pressure gauge till from 12 to 15 pounds pressure is reached.
- (f) Adjust the heat source so that the pressure neither rises nor falls during at least thirty minutes (for quart jars and smaller sizes).
- (g) At the end of the time of sterilizing, turn off the heat and *allow the pressure to subside completely*. Do not open the air vent until the pressure is down.
- (h) Open the air vent.
- (i) Remove the lid and the food vessels and tighten any loose covers.

Acid foods like tomatoes need not be processed as long as neutral foods like corn, peas and spinach, since the combination of acid and

heat is more rapidly bactericidal than heat and a neutral reaction. Foods in large masses, like meat, should be processed longer in order to allow the heat to penetrate the interior. All foods should be covered with fluid. Don't fill jars over three-quarters full, or they are apt to boil over.

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## CHAPTER 33

### THE SPHERICAL BACTERIA. I. MICROCOCCACEAE

A FEW species of spherical or oval forms of bacteria are found in the orders Thiobacteriales and Chlamydobacteriales but all of the cocci of major importance in industry, agriculture and medicine are included in the order Eubacteriales. Here, except for the few coccial forms found in the genus *Nitrosococcus*, the important families of cocci are included in three well defined groups, namely Streptococceae, Micrococcaceae, and Neisseriaceae.

All the organisms of these groups of cocci are rounded or some modification of the spherical form (oval, elliptical, and so on). None forms spores and none is motile. Most of the cocci are aerobic or facultative although a few are strict anaerobes.

The cocci are divided into several subgroups on the basis of the form and arrangement of the cells (see Chapter 4). Among the important genera are those in the family of Micrococcaceae, and we shall turn our attention first to these.

#### FAMILY MICROCOCCACEAE

The organisms of this family are usually nearly spherical in form. Chain formation is not characteristic of these organisms, although occasionally as many as three or four cells will cling together in a row temporarily. Odd shaped clumps are the rule. Pairs of micrococci are frequently seen, especially in young broth cultures. Various other arrangements resulting from cell division in two or three planes are characteristic. The family includes some pathogenic species (*Staphylococcus aureus*, *S. albus*, *Gaffkya tetragena*) but most of the Micrococcaceae are harmless saprophytes.

**General Characters.**—All of the organisms of the family Micrococcaceae grow well aerobically at temperatures of from 30° to 37° C. on simple artificial media, such as extract agar, although as a rule the growth of the saprophytic species is definitely more luxuriant than that of the parasitic types such as the true staphylococci. A large majority form opaque, moist colonies colored with white, yellow or red pigments; various shades of red and orange are especially common among the saprophytic types. All are gram-positive.

**Spatial Arrangement.**—The spatial relationships of the Micrococcaceae are best seen by observing broth cultures in hanging



drop preparations. Here the tetrads of the *Gaffkya* and the cubical packets of the *Sarcina* are readily distinguished, as are frequent pairs and small, irregular clumps of *Micrococcus* and *Staphylococcus*. Large masses of mucilaginous capsular material may often be seen enclosing the tetrads of *Gaffkya*.

**Classification.**—The classification of the Micrococcaceae is difficult. Probably the simplest procedure would be to classify in the genus *Gaffkya* those species which divide in two planes at right angles, producing groups of four cells or tetrads (hence *Gaffkya tetragena*, the type species of the genus); to include in the genus *Sarcina* those which divide in three planes at right angles, forming cubical packets; and to assign to the genus *Staphylococcus* all those species forming irregular clusters of cocci suggestive of bunches of grapes. However, it is common practice to speak of the nonpathogenic cocci of the last group as the genus *Micrococcus*.

**Role of the Micrococcaceae in Nature.**—Except for the staphylococci and *Gaffkya tetragena*, which are often pathogenic, the Micrococcaceae as a family are of importance (as far as is known at present) mainly to the student who views bacteriology as a botanical and biological science. They are found widely distributed in milk, soil, dust and water, especially where putrefaction is in progress. They are among the commonest contaminants in laboratory cultures. Their function in nature seems to be that of accessory putrefactive organisms, or scavengers, since many have the ability to digest proteins like gelatin and casein and to attack various carbohydrates and other organic substances. A few species (*M. cereus*, *M. perflavus*, *M. caseolyticus*) may be of commercial importance in the ripening and flavoring of cheese since they attack casein and lactose with the production of aromatic substances having pleasing flavors, and some produce various undesirable conditions such as "ropy milk" (*M. viscosus* and *M. cremoris-viscosi*).

Most of them produce brilliantly colored colonies but the function of their pigment is not well understood. It seems to have neither photosynthetic power, like chlorophyll, nor respiratory function like hemoglobin. The pigments of the cocci are presumably harmless. Pigment formation is often a rather variable characteristic.

Occasionally one or another species of *Micrococcus* is found apparently causing a pathological condition but may, in such a case, be regarded as what Theobald Smith called an "opportunism";

i.e., an organism (of any sort) which is able to take advantage of some special or unusual combination of circumstances (such as the weakened condition of a patient or an old ulcer) favorable to its development.

**Genus Staphylococcus.**—These organisms have the general properties of other micrococci, forming irregular clumps, being gram-positive and growing well on ordinary laboratory media.<sup>1</sup> In general, the individual cells of staphylococci are smaller than those of the other Micrococcaceae (Fig. 259), and staphylococci generally ferment lactose and liquefy gelatin. However, these properties are neither constant nor exclusive among staphylococci.

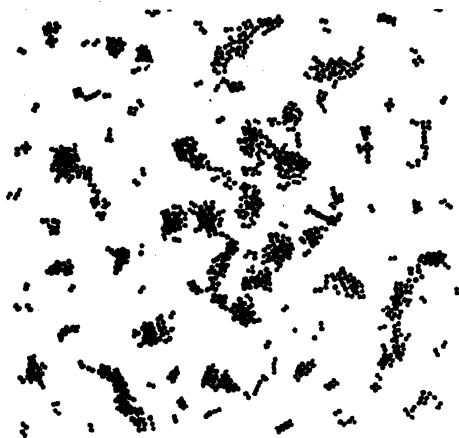


Fig. 259.—*Staphylococcus aureus* from 24-hour culture on agar, stained with safranin ( $\times 900$ ). (Ford.)

and it is sometimes very difficult to make a distinction between staphylococci and micrococci, as many strains of the genus *Staphylococcus* possess properties of the genus *Micrococcus*, and vice versa, especially the white varieties of both genera. True staphylococci, especially *S. aureus*, often produce very hemolytic colonies when cultivated on the surface of blood agar.

The true staphylococci are regarded as parasites and are usually to be found only on the skin or mucous membranes of the animal body, especially of the nose and mouth, where they often occur in enormous numbers even under "normal" conditions. Two principal species may be mentioned: *S. aureus*, distinguished by its golden-yellow pigment and notorious as the cause of mastitis

of cows, boils, carbuncles, and internal abscesses; and *S. albus*, recognized by its paper-white pigment, opaque colonies, and universal presence on the human skin. The golden-yellow species are regarded as much more dangerous than the white species, although the latter often cause infections of the skin and occasionally severe abscesses.

*Types of Staphylococci.*—Julianelle<sup>2</sup> made extracts of staphylococci, containing specific carbohydrates analogous to the group-specific carbohydrates of streptococci and other organisms. He described two serological groups, which are referred to as "A" and "B." Group A staphylococci are generally found related to disease processes, while group B are usually regarded as having little or no pathological significance. Group B forms might be classed in the genus *Micrococcus*.

The serological properties are correlated fairly well with biochemical properties.<sup>3</sup> Thus, group A strains *generally* ferment mannite, and produce a variety of pathological effects. There are various names for the toxin (or toxins) on which these effects depend. It is not entirely clear just how many separate toxins there may be. The effects have been variously described as due to (1) *lethal toxin* (kills animals, for example rabbits, very quickly, [a few minutes] after intravenous injection); (2) two sorts of *hemotoxin* (alpha hemotoxin, which hemolyzes rabbit and sheep erythrocytes and beta hemotoxin, which does not hemolyze rabbit cells); (3) *dermotoxin* (produces a necrotic area in the skin when injected intradermally); (4) *coagulase* (brings about coagulation of citrated blood); (5) *fibrinolysin* (digests fibrin); (6) *leukocidin* (kills leukocytes); (7) *enterotoxin* (produces acute gastro-enteritis when ingested by human beings and some of the lower animals).<sup>4</sup>

Most of these activities of staphylococcus cultures or culture filtrates are easily demonstrated. Animal injections serve for the demonstration of the lethal and dermonecrotic factors, and hemolysis and leukocytocidal activities are readily demonstrated by mixing suspensions of the appropriate cells in test tubes with the tested culture and observing the effect microscopically. The fibrinolysin test is similar to that used for streptococci. The test for coagulase is a simple one, and valuable because coagulase production seems to be most closely related to virulence or pathogenicity of the cocci.<sup>5</sup> All that is necessary is to mix, in a small tube, a loopful of young growth from an agar culture of the tested organism and 0.5 cc. of freshly prepared citrated or oxalated

human plasma diluted about 1:3 with saline or broth. Coagulation will occur in an hour or so at 37° C. It sometimes progresses slowly.

There is some evidence that the lethal, dermonecrotic and alpha hemotoxic effects are due to the same factor. However, there is conflicting evidence on this point, so that definite statements cannot be made. Coagulase and fibrinolysin seem entirely independent substances, and leukocidin may be. The enterotoxin is a separate and distinct fraction of the toxic principle of staphylococci. Group B strains usually do not possess any of these properties. However, there are many exceptions and irregularities so that these divisions are not very clear-cut.

Verwey has shown that strains of groups A and B may be roughly differentiated by means of the electrophoresis apparatus, the group A strains generally moving slowly, the group B strains rapidly.<sup>3</sup>

*Staphylococcus Enterotoxin.*—Many strains of staphylococci not differentiated by any known property, and belonging to either group A or B produce an extracellular substance, *enterotoxin*, which causes acute gastro-enteritis when swallowed with food. Poisoning by ingestion of staphylococcus enterotoxin results in nausea, vomiting, diarrhea and prostration. It usually follows ingestion of the toxin within a few hours and lasts for only a few hours or a day.<sup>6, 7</sup> It is seldom fatal, but a few deaths have been reported.<sup>9</sup> It is a relatively stable poison, and may be separated from the lethal, skin-necrotizing, and hemolytic toxins by heating mushy agar (0.2 percent agar) culture filtrates at about 95° C. for ten to twenty minutes. This does not destroy the enterotoxin, but destroys the other toxins mentioned. Tests for enterotoxin are usually made by injecting the heated filtrates into kittens, which then show such symptoms as dizziness, vomiting, defecation and general weakness.

The staphylococci grow in many foodstuffs, notably the cream used in cream-puffs,<sup>8</sup> in milk<sup>9</sup> and in fish cakes, although precooked foods of many sorts—meats, pies, salads, etc.—have been incriminated at various times. Starchy foods in general seem to favor bacterial food poisoning.<sup>10</sup> The staphylococci appear to gain entrance to the foods from boils or abscesses on the skin or in the nose or mouth of food handlers, and the enterotoxin forms as they grow. If foods are not promptly refrigerated after cooking, considerable toxin may accumulate. Subsequent inadequate heating may kill the staphylococci yet spare the toxin, since the latter is

rather heat-resistant.<sup>11</sup> Refrigeration of bakers' goods containing cream, and similar precautions with other foods, are desirable.

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## CHAPTER 34

### THE SPHERICAL BACTERIA. II. THE STREPTOCOCCI

AS THE NAME implies, streptococci grow in chains (Fig. 260). Sometimes chains of only two, three or four cells occur; in other species very long chains of several thousand cocci may be observed. Some interesting details of how streptococci hang together have been revealed with the electron microscope (Fig. 261). *Chain formation can be accurately observed only by cultivating the streptococci in pure culture in broth and observing them in stained smear or hanging-drop preparations.* All are gram-positive (although some of them appear to be gram-positive only when cultivated in blood or serum medium), and many of them are pathogenic. All grow on infusion agar or in infusion broth, but some of them, especially the pathogens, grow better if blood or serum be added. Some are quite proteolytic, especially certain of the pathogenic beta hemolytic type which actively digest dead tissues and fibrin, and streptococci

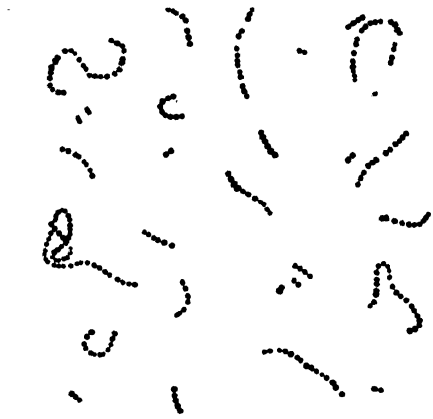


Fig. 260.—Typical streptococci ( $\times 900$ ). (Ford.)



Fig. 261.—Electron microscope picture of streptococci ( $\times 20,000$ ). (Zworykin.)  
Note the thin protoplasmic filaments connecting the cells. Compare with Figure 260.

of the vagina and intestine which liquefy gelatin. All ferment dextrose without formation of gas. Three genera are included in this tribe: *Diplococcus*, *Streptococcus* and *Leuconostoc*.

TABLE X  
SOME DIFFERENTIAL CHARACTERS OF STREPTOCOCCI

Group Designation	Tube Hemolysis§	Action on Blood-agar Plate	Growth at		Survival of 60° C.	Growth in			Representative or Important Species or Varieties	Lancefield Group	Ferments						Attacks		Human Fibrinolysin	Bile-soluble	
			10° C.	45° C.		NaCl 6.5%	pH 9.6	0.1% meth. blue.			Lact.	Man.	Sal.	Tre.	Sorb.	Acid in Dextrose Broth	Sol. Hip	Gel.			
Beta-type hemolytic streptococci or "Str. hemolyticus" (pyogenic group)	(-) +	β	-	-	-	-	-	-	Str. pyogenes Str. agalactiae Str. equi "human" ("animal")	{ A F B C	+	+	+	+	-	little much little mod. little	+	+	+	+	
Alpha-type hemolytic streptococci "Str. viridans"	-	α some γ	-	+	some +	-	-	-	Str. salivarius Str. thermophilus* Diplococcus pneumoniae†		+	+	+	+	-	much much much	+	+	+	+	
Gamma or indifferent type streptococci or "lactic streptococci"	-	γ	-	-	+	-	-	+	Str. lactis† Str. cremoris‡		+	+	+	+	+	much much	-	-	-	-	
"Enterococci"	-	α β γ	-	-	+	-	-	-	Str. faecalis Str. liquefaciens Str. zymogenes	D*	+	+	+	+	+	-	much much	-	-	-	-

\* Distinguished especially by (a) growth at 50° C. and (b) failure to ferment maltose, from all other streptococci in this list. Is a great nuisance because resistant to pasteurization.

† Does not grow at 45° C. and always produces alpha type colonies.

‡ These are differentiated by the ability of *Str. lactis* to grow at 40° C. and in media with a salt concentration of 1 per cent or a pH of 9.2. *Str. cremoris* cannot grow under these conditions.

§ Beta hemolysis in blood agar.

§ Hemolysis of saline red-cell suspensions by young serum-broth cultures when the two fluids are mixed in about equal amounts.

**Relations of Various Groups of Streptococci.**—The first two genera of this tribe (*Diplococcus* and *Streptococcus*) are very closely related in many important respects, namely, morphology, chain formation, capsule production, and preference for blood or serum media. Both are found in the normal mouth and upper respiratory tract as well as in the intestinal tract, etc. It seems of doubtful utility to classify them in separate genera. However, members of the genus *Diplococcus* are differentiated from many species of streptococci by several physiological peculiarities. Most striking of these properties of *Diplococcus* are solubility in bile, ability to ferment inulin, production of lobar pneumonia in human beings, possession of large, easily stained capsules, and serological behavior. The members of the third genus of streptococci (*Leuconostoc*) are found in situations unrelated to a parasitic existence or pathogenic activity, like vats in sugar refineries and fermenting sauerkraut and are of considerable importance in the dairy industry because they grow in milk, butter and cheese, imparting flavors to the butter and cheese.

**Classification of the Genus Streptococcus.**—One of the most difficult problems in bacteriology is the systematic subdivision of a family or tribe into genera and species, and the difficulty of the problem is well illustrated by the streptococci. Since many of the methods used for studying streptococci are now being adapted to other groups of organisms, the problem as it relates to the streptococci will be discussed in some detail.

For convenience an outline, adapted from Sherman,<sup>1</sup> of the more general relationships is placed here for reference when the reader finds the details oppressive.

Additional clarification will be found in Table XI.

Differential criteria assigned to subgroups such as genera and species should be fairly constant, and so definite as to be always readily observed. As to the number and nature of the differential characters which may be required of an organism in order that it may be distinguished as belonging to a species or genus, there is no agreement. This is particularly so among the streptococci, so that species differentiations are somewhat confused. For our purposes, perhaps the simplest procedure will be to discuss the currently used differential criteria and describe the so-called species (or races or varieties) as they are differentiated by the various tests applied to them.

**Blood-agar Plates.**—Possibly the most generally useful method for primary differentiation of streptococci (genus *Streptococcus*) is



cultivation in blood-agar pour-plates.<sup>2</sup> The differential criterion is the effect of the colonies of streptococci on the red blood cells in the agar. In order to determine this, a tube containing about 15 cc. of melted infusion agar, cooled to about 45° C. (still fluid), is inoculated with a loopful of pus or broth culture or other material containing the desired streptococci, and about 5 percent sterile blood is added and well mixed with the agar. The mixture is poured (Fig. 262) into plates and incubated at 37° C. for 24 hours.

TABLE XI

*Interrelations of Streptococci**Blood agar types*

*Beta* hemolytic (clear zones). Grow well only at 37° C.

## Lancefield groups

(Almost all of these  
are definitely patho-  
genic.)

- |   |  |
|---|--|
| { | <p>A. <i>Str. pyogenes</i>, etc.<br/>Griffith's agglutinative types 1 to 30 +, corresponding with types determined by Lancefield's M substance.</p> <p>B. <i>Str. mastitidis</i> of bovine mastitis, and other strains of bovine origin. Double-zone beta type.</p> <p>C, D, E, etc.</p> |
|---|--|

*Alpha* hemolytic (inner green zones) ("viridans"). Grow well at 35° to 40° C., not at 10° C. Many animal and human pathogens. *Str. salivarius*, *Str. equinus*, etc. A few species are said to produce both alpha and gamma colonies.

*Gamma* (no visible zone) (indifferent or nonhemolytic).

1. Lactic group (*Str. lactis*, etc.). Grow well at 10° to 30° C.
2. Pathogens, much like pathogenic alpha type streptococci.

*Enterococcus group*: inhabit human and animal intestinal tract. This group includes species which produce alpha, beta or gamma zones in blood agar. These all grow well at 10° to 45° C., under cultural conditions too adverse for other species. In general the enterococci are relatively rugged organisms.<sup>1</sup> They attack carbohydrates vigorously, and some species are proteolytic. A few are pathogenic.

**Types of Streptococci as Differentiated on Blood Agar.**—Three main types of streptococci are recognized, depending on their action in blood-agar plate cultures prepared as just described. There are two *hemolytic* types, *alpha* and *beta*, and one *nonhemolytic* or indifferent type known as the *gamma* type (Table XI).

*Alpha Type*.—Colonies of alpha-type hemolytic streptococci in blood-agar pour-plates prepared as described above, when examined with the low power of the microscope are seen to be surrounded by a zone of hemolysis and also by a zone of discolored erythrocytes close in around the deep colonies, the latter clearly visible only with the low power of the microscope. These erythrocytes have a green or brownish-green color. Beyond this

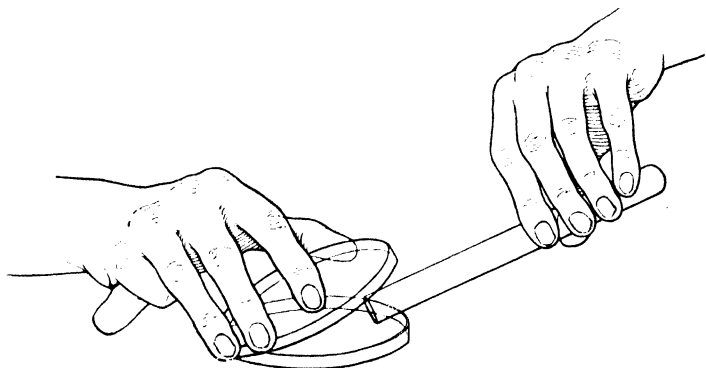


Fig. 262.—Method of pouring a plate. The cotton plug can be held as shown, or can be grasped between the ring and little finger

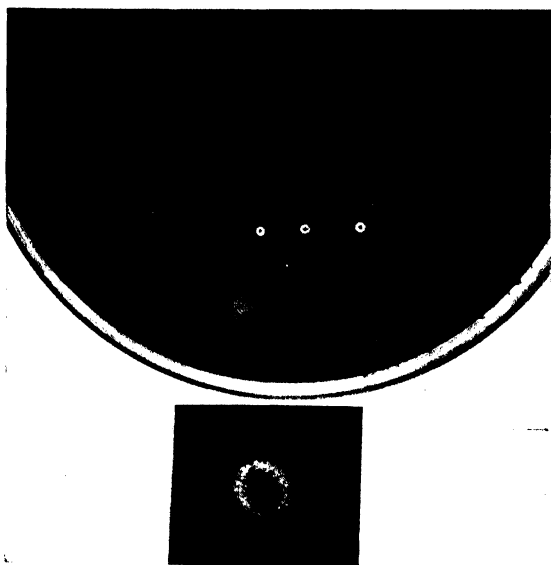


Fig. 263.—Natural size photograph of blood agar plate showing colonies of *alpha*-type hemolytic streptococci (*Streptococcus viridans*). Each colony is surrounded by a small clear zone. In the center, close around the colony, is a zone of greenish or brownish corpuscles. Sometimes this zone is so wide as to mask the clear part. Often it is so narrow as to be visible only with the microscope as shown in the small insert. The colony may then be mistaken for a true beta type *Streptococcus* colony. (Courtesy of Dr. J. Howard Brown, Johns Hopkins University.)

inner ring of discolored cells the outer zone of clear hemolysis may be of great or small extent, and may sometimes be so small as to be invisible, but usually widens on refrigeration of the plate.

When viewed with the naked eye, alpha-type colonies having a *wide* zone of hemolysis are sometimes mistaken for colonies of hemolytic streptococci of the beta type, especially if the inner, green zone be very narrow, as often happens. *Only the use of a*



Fig. 264.—Natural size photograph of blood agar plate showing colonies of beta-type hemolytic streptococci. Each colony is surrounded by a perfectly clear zone in which all the blood corpuscles have been completely destroyed. The insert shows one of the colonies much enlarged. (Courtesy of Dr. J. Howard Brown, Johns Hopkins University.)

*microscope can be relied upon to make the distinction and only colonies which are deep in the agar are always thus characterized, surface colonies sometimes producing deceptive appearances. The green-producing varieties of hemolytic streptococci (hemolytic streptococci of the alpha type) are often loosely called Str. viridans (Fig. 263). Sometimes, upon alternate refrigeration and incubation, several concentric zones of hemolysis and greenish discoloration*

may be produced. These must not be confused with the double-zone beta type streptococci described below.

*Beta-type Streptococci.*—The hemolytic zones of streptococci of this type in blood-agar plates are seen to be entirely clear and free from any intact erythrocytes. Such streptococci are loosely spoken of under the general term of “streptococcus hemolyticus.” They may more accurately be termed *hemolytic streptococci of the beta type* (Fig. 264).

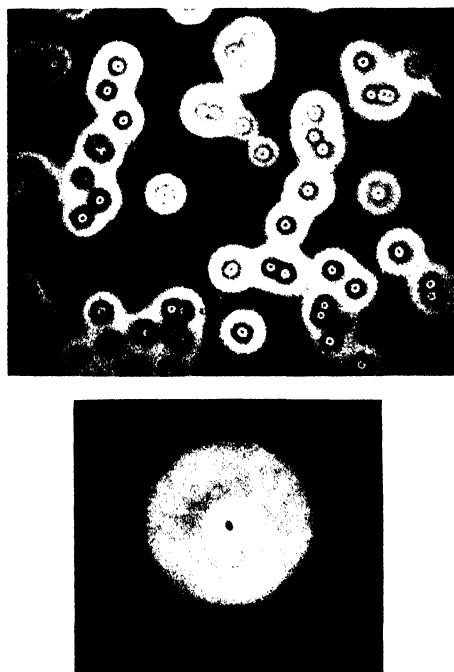


Fig. 265.—Upper, double-zone beta-type hemolytic streptococcus colonies in blood agar, incubated 24 hours at 37° C. and then refrigerated overnight. Lower, single colony enlarged 8 times. (Brown, Jour. of Bact., Vol. 34.)

*Double-zone Beta-type Streptococci.*—A very interesting subdivision of the beta-type streptococci is seen in the double-beta-zone-producing subtype. It was first described by Brown in 1937.<sup>3</sup> Certain species, almost exclusively of bovine origin, after producing a zone of hemolysis like that of other beta-type streptococci, on standing at room temperature or on refrigeration produce a second ring of hemolysis separated from the first by a ring of red

erythrocytes (Fig. 265). All double-zone beta-type hemolytic streptococci so far described have belonged to Lancefield's group B (see below) but not all group B strains are known to produce double zones.

*Gamma Type*.—The third type of appearance of colonies of streptococci in blood-agar plates is a complete absence of any visible change in the blood cells surrounding the colony. Such streptococci are said to be of the *gamma type*. This type of streptococci has not been very thoroughly studied.

**Other Subdivisions of Streptococci**.—Each of the types of streptococci distinguished, as described above, according to its action on blood agar may be again divided, on the basis of still other properties, into what are variously termed divisions, varieties or species, or subspecies, depending on one's conception of the meaning and scope of these terms.<sup>4</sup> Specific names are given to some of the better known varieties of each division, on the basis of source or other properties, and this is a very convenient though taxonomically dubious practice. For example, when beta-type hemolytic streptococci were first discovered, it was customary to name them according to the disease they were thought to cause. *Str. pyogenes* (pyogenes = pus-producing) was a name early given to streptococci causing suppurative infections of various parts of the body. This name still holds and *Str. pyogenes* is the type species of the genus. Streptococci from erysipelas were called *Str. erysipelatis*, those from scarlet fever were called *Str. scarlatinae* and so on. However, it has since been found that the beta-type hemolytic streptococci causing one disease can often cause other diseases interchangeably with other beta-type hemolytic streptococci.

Streptococci found in patients during septic sore throat epidemics and having very heavy and constant capsules, have been termed *Streptococcus epidemicus*. However, since capsules are not confined to septic sore throat streptococci, and since these streptococci do not seem to be any more specific for this than for any other streptococcus disease, this species name also is of doubtful validity. By proper procedures, capsules can be demonstrated on most of the pathogenic beta-type hemolytic streptococci (Fig. 266). The tendency at present is, therefore, to drop the disease names and refer simply to *hemolytic streptococci of the beta type*, stating the source or, better, the Lancefield group (see page 546).

**Fermentative Varieties**.—Attempts to establish species of streptococci on the basis of fermentation reactions have led to great confusion. Many of the "species" established by this method

are invalid because various fermentative types occur in any of the groups established by other means. Only a few specific fermentation tests are of use as applied to some of the finer distinctions among varieties of these organisms established on other grounds.

**Serological Grouping of Hemolytic Streptococci.**—As far as the beta-type hemolytic streptococci are concerned, serological grouping has proved most informative. For years, attempts were made to distinguish between groups of streptococci, especially the



Fig. 266.—Streptococci of the beta type stained by Hiss's method for capsules  
1, Group A; 2, group B; 3, group C. (After Hobby and Dawson.)

pathogenic types, on the basis of their agglutination reactions. For example, it was found by Griffith and others that most scarlet fever streptococci are agglutinated by one or another of several "type"-agglutinating sera prepared with various "types" of scarlet fever streptococci; most erysipelas streptococci were agglutinated by erysipelas-agglutinating serum, and so on. These were often spoken of as "Griffith's types." However, there has been much dissatisfaction with this method because of technical difficulties

and failure of many types to correlate with clinical data.<sup>5</sup> However, more recent studies show that the agglutinative typing of certain streptococci is of great value epidemiologically.<sup>6</sup>

**Lancefield Groups.**—The most useful means of studying beta-type hemolytic streptococci have been developed during the last decade by Lancefield at the Rockefeller Institute.<sup>7</sup> She made extracts of massive cultures of different strains of these bacteria by means of hot N/20 HCl. These extracts contain specific, somatic, antigen substances. The clear extracts are used in precipitin tests. The precipitin-containing serum used in these tests is prepared by injecting rabbits with the sedimented cocci from selected, 18-hour-old broth cultures treated with 0.2 percent formaldehyde.

By making tests<sup>8</sup> with antisera and extracts of beta-type hemolytic streptococci from various sources, it was found that there were fairly distinct precipitin *groups* of streptococci with respect to origin, and Lancefield designated these groups by letters of the alphabet. It is essential that the student differentiate between Lancefield *groups* of streptococci and hemolytic *types*, as well as Griffith's agglutinative types (see Table XI).

It must be remembered also that Griffith's types and Lancefield's grouping apply *only to beta-type hemolytic streptococci*. The alpha and gamma types have not been found to fit into these schemes, although data are accumulating concerning this aspect of the problem and some strains of the alpha type have been found to react with group-specific sera of the beta type.

**Group A Streptococci.**—All strains of group A yield a carbohydrate (in the extraction process described) which acts as the precipitation reagent in the precipitin test. It is called a "C" (Carbohydrate) substance. The C substance is the common antigenic component in all members of group A.

Biological properties distinguishing group A streptococci from other groups are shown in Table X. Special attention is called to their reactions on the substances trehalose and sorbitol, the former of which is fermented, the latter not. The special value of this fact will be pointed out later (see page 550).

Subgroups or specific *types* among the group A strains (corresponding to the agglutinative types of Griffith previously mentioned) can also be distinguished by the precipitin test, using as antigen a protein "M" substance extracted by a process similar to that used to extract the "C" substance. This M substance is present in certain variants called *matte* variants.

**Matte and Glossy Variants.**—An interesting and important vari-

ation of the concept of R and S variation is seen in the *matte* (rough) and glossy (smooth) colony types of beta hemolytic streptococci described by Todd and Lancefield.<sup>9</sup> Here virulence is usually associated with the matte variant. The *type-* (not group-) specific protein (M substance) in group A matte strains seems to be absent from the glossy variants. It would appear that type specificity, virulence and the M substance are closely related in streptococci, a relationship suggestive of that existing between Vi antigen, virulence and specific-bacteriophage type-specificity in typhoid bacilli.

Another substance (T substance) also appears to confer the *same type* specificity as the M substance on streptococci, but seems unrelated to virulence, since it occurs in both matte and glossy variants.<sup>8, 9</sup>

Such phenomena, although most thoroughly studied in relation to streptococci, are probably of broad biological significance and must be thought of as probably typifying similar relations existing, but as yet undiscovered, among many entirely unrelated species of bacteria and possibly higher plants as well.

*Streptococcus pyogenes*.—The group A streptococci are primarily human pathogens. They are best represented by the variety or species known as *Str. pyogenes*. This name was given to the streptococci found in infected wounds, when they were first isolated and studied by Rosenbach in 1884. This species is the common cause of "blood-poisoning," mastoiditis, puerperal sepsis, scarlet fever, septic sore throat and erysipelas, and is one of the most deadly and dangerous pathogens known. It has the characters of all group A streptococci, and typically ferments lactose and salicin but not mannite. Some strains ferment other substances and are often called by other species names. It was shown by Foley, Getting, et al., in 1943<sup>10</sup> that scarlet fever strains may produce outbreaks of food poisoning by developing an *enterotoxin* substance as a result of their proteolytic action on meats (see section on staphylococci, page 535).

**Pathogenic Action of Beta-Type Streptococci of Group A.**—Among the commonest diseases caused by beta type streptococci of group A are infections of the tonsils and throat, sinuses, lungs and blood. Two representative clinical manifestations of throat infection are scarlet fever and septic sore throat. A discussion of these diseases will illustrate a number of principles of host-parasite relationship in general, whether the parasite be streptococci or other bacteria.



*Scarlet Fever and Septic Sore Throat.*—These diseases are both due to hemolytic streptococci of the beta type, Lancefield's precipitin group A. The organisms gain entrance to the throat by direct contact with a patient, or infected dust, milk, or some other means of transmission, find conditions in the tonsils or in the adjacent tissues favorable for their growth, and proceed to establish themselves, giving rise to the diseases mentioned by giving off toxins and by invading the tissues and blood stream.

There are several points of interest about these organisms and the diseases they cause. First, any one of a number of cultural varieties or agglutinative (Griffith) types of group A streptococci may cause either disease. There is thus a certain lack of specificity as to etiology. Second, septic sore throat is the same disease as scarlet fever, differing only in the absence of a rash.<sup>11</sup> The rash in scarlet fever is due to the susceptibility of the patient to the *exotoxin* (*erythrogenic* or *rash-producing toxin*) of these organisms. The immunity to the scarlatinal erythrogenic toxin is very durable. Persons who have developed an immunity to this exotoxin may still contract *infection* with the streptococci but *do not develop the rash*. In such persons the invasion of the tonsils (septic sore throat) and adjoining tissues (ear, mastoids, etc.) goes on just the same but there is *no rash*.

Scarlet fever antitoxin has been prepared according to methods devised by a German scientist, Moser, in 1902, and by the Drs. Dick<sup>12</sup> in this country. It is similar to diphtheria antitoxin. This combats the *exotoxin* but offers little protection against infection with the streptococci. A very satisfactory preparation has been prepared by Veldee.<sup>13</sup>

*Other Pathogenic Actions of Hemolytic Streptococci.*—Hemolytic streptococci of Group A (also groups B, C, F and G) are associated with many acute, inflammatory, pyogenic conditions.<sup>16</sup> The number of leukocytes in the blood stream increases greatly during such infections, although no such *local* aggregations of them occur as in *Staphylococcus* infections. Like the staphylococci, all group A beta-type streptococci produce hemotoxin, leukocidin, exotoxin and endotoxin. Streptococci of groups A, C and G produce an enzyme capable of dissolving human blood clot (fibrin) as well as a trypsin-like enzyme digesting dead tissues.<sup>14, 15</sup> It is likely that these enzymes enable them to overcome one of the important defensive measures of the human host and to invade tissues and blood vessels more rapidly. Infected persons often develop a counter-enzyme or antibody called antifibrinolysin.

*The Dick Test.*—The Dick test, devised by the Drs. Dick of Chicago, is performed by injecting into the skin a very tiny amount of group A streptococcus erythrogenic toxin. If, in about twenty-four to thirty-six hours, a red spot at least 1 cm. in diameter appears at the site of injection, it is inferred that the person has not sufficient antitoxin in his blood to protect him against even a minute dose of this particular toxin and is therefore likely to develop a rash (scarlet fever) when infected with strongly toxigenic strains. A similar test, called the Schick test, is used in diphtheria and is discussed in connection with the diphtheria bacilli (see page 669).

*Septicemia* is often caused by beta-type streptococci of group A which produce much endotoxin. The endotoxin seems to confer a greater aggressiveness than is common to other groups of streptococci. Such strains invade the tissues and blood stream readily, causing erysipelas, "blood poisoning" or septicemia, malignant and acute sore throat (septic sore throat) or tonsillitis, certain forms of pneumonia, meningitis (inflammation of the coverings of the brain and spinal cord), peritonitis (inflammation of the peritoneal or abdominal cavity), pleurisy, and puerperal sepsis (child-bed fever or blood poisoning). However, any of these diseases may, at times, be caused by streptococci of the other groups.

The use of serum against infections in which the organisms are of the predominantly endotoxic sort is less efficacious than in scarlet fever, partly because the endotoxins, being intracellular, are protected from the action of the serum.

**Group B Streptococci.**—The organisms of this group resemble those of Group A in possessing in common a group-specific "C" substance which is chemically different from that of group A cocci. Group B cocci also are separable into "types" by means of a second substance; but in group B this second substance is a carbohydrate and not a protein like the "M" substance of group A. It is spoken of as "S" substance.<sup>17</sup>

The group B streptococci differ from those of all other Lancefield groups in hydrolyzing sodium hippurate. The very striking property of double-hemolytic-zone production by group B streptococci has been referred to above (page 543). Another distinctive property is that of usually producing a more strongly acid reaction (pH around 4.6) after five days growth in 1 percent dextrose broth than group A, C or G strains. Hemolytic streptococci of group B are usually of bovine origin but are occasionally found in human infections. *Str. mastitidis*, an important member of

group B, is of particular interest to the farmer because it is one of the common causes of mastitis in cows and because it is sometimes confused with dangerous group A streptococci which may also cause mastitis in cows and, when discharged in large numbers in the milk, may produce widespread outbreaks of scarlet fever and septic sore throat.<sup>18-21</sup> The group A streptococci gain entrance to the udder from the hands of milkers who have the organisms in their throats. When the infection of the udder due to either group of cocci is acute, the streptococci may be found in the milk by inoculating melted infusion agar with diluted milk and pouring it, mixed with blood, into Petri plates as already described. The hemolytic colonies appear after twenty-four hours' incubation at 37° C.

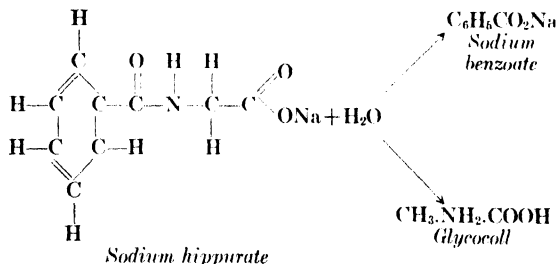
**Differentiation of Group A and Group B Streptococci.**—The bacteriologist, investigating the cause of an epidemic, is often faced with the problem of determining whether hemolytic streptococci in milk are of the usually harmless bovine (group B) or of the dangerous, human (group A) sort. This is not difficult. Pure cultures isolated from the milk by means of blood-agar plates are planted in a tube of broth containing 1 percent dextrose, and in broth containing 1 percent sodium hippurate. Differentiation may be made on the basis of ability to hydrolyze sodium hippurate.\*

\**Test for hydrolysis of sodium hippurate:*

1. The medium should be freshly prepared. In sterilizing apply not over 10 lbs. steam pressure for not over 15 minutes in order to avoid hydrolysis of the hippurate.
2. To 2 cc. of the culture, after 48 hours' incubation, add 0.4 cc. of the following reagent:

FeCl <sub>3</sub> .....	12 gm.
HCl (2%).....	100 cc.

3. The development of a bulky, brownish precipitate of ferric benzoate,



which remains in almost undiminished amount (around one half the volume of the fluid) for an hour or more, indicates hydrolysis. Small amounts of non-specific precipitate often appear.

4. Group B streptococci produce hydrolysis, others do not.

or by means of the precipitin test, following Lancefield's or Fuller's procedure.<sup>22</sup> Double hemolytic zones, when observed, are strongly indicative of group B strains.

**Group C Streptococci.**—The group-specific substance in these cocci is probably not a carbohydrate although its exact nature is not entirely clear. The group is of a dual nature with respect to origin, containing (1) strains of human origin culturally indistinguishable from group A cocci. They are not such dangerous pathogens. (2) Strains from various animal sources. The latter rarely occur in human infections and may be divided into two subgroups on the basis of fermentation reactions. One, represented by the species called *Str. equi* because it is found especially in diseases of horses, ferments neither sorbitol nor trehalose. The second, occurring in diseases of horses as well as of animals such as cattle, guinea-pigs, rabbits and ferrets, ferments sorbitol but not trehalose. The group C strains of human origin ferment trehalose but not sorbitol. They also produce human fibrinolysin, which the animal strains do not. Other characters are shown in Table X.

**Group D Streptococci.**—These streptococci, like those of group C, possess a common antigen among themselves that is probably not a carbohydrate. They differ markedly from cocci of groups A, B and C in a number of important biochemical and physiological properties which are shown in Table X.

They are found mainly in human or animal feces, milk and cheese and are probably without pathological significance, although one species (*Str. zymogenes*) has, on occasion, apparently been implicated as the causative agent in certain human infections.

Because of their relationship to the intestinal contents, they are sometimes included among "enterococci." Some of them are of great industrial importance especially as they occur in milk and give flavors to butter and cheese. There are a number of serological and biological types.

**Group G Streptococci.**—These strains are possibly related antigenetically to some group C strains, and closely resemble group A strains in important biochemical properties. They are generally not highly pathogenic, but vary in this respect. They are frequent in human infections.

**Groups E, F, H and K.**—These groups contain such small numbers of strains that they will not be discussed in detail. They do not appear to have any special pathological significance, but

have been found occasionally in animals and human beings, and also in dairy products.

**Alpha and Gamma Types.**—The alpha-type hemolytic (or “viridans”) streptococci and the gamma or nonhemolytic varieties may be considered under three subheadings: the viridans streptococci proper; the lactic group, common in milk; and the so-called enterococci. The subdivisions are not so clearly differentiated as might be indicated; that is, some of the alpha type species appear to form both alpha and gamma colonies, and some gamma species are found in the so-called viridans group as well as in the lactic group and among the enterococci. For this reason alpha and gamma types from all sources are included in one discussion.

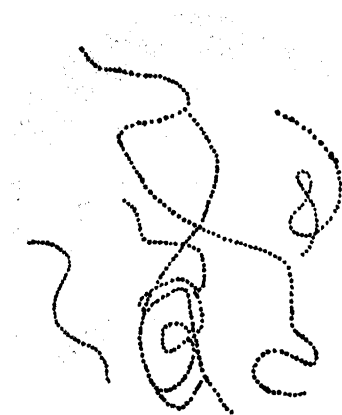


Fig. 267.—Hemolytic streptococci of the alpha type (*Str. salivarius*), cultivated in broth from an abscessed tooth. Very long chains ( $\times 900$ ).

**Viridans Streptococci.**—The first of the subdivisions mentioned above comprises the common streptococci of the alimentary tract. These are represented by *Str. salivarius* of the human mouth (Fig. 267). The colonies of this species in blood agar are definitely of the alpha type. The effects of *Str. salivarius* on lactose, mannite and salicin are like those of *Str. pyogenes* which shows how little differential value is to be placed on fermentative properties of this sort. *Str. salivarius* might be called the “type species” of the “viridans group.” Similar varieties are found in the intestinal tract of the horse (*Str. equinus*) and cow (*Str. bovis*). A related species occurs in milk (*Str. thermophilus*.) The last of these, as the name implies,

is resistant to, and grows well at, elevated temperatures (around 60° C.). It gives much trouble to commercial milk pasteurizers because, since it is not destroyed by pasteurization and grows to large numbers in pasteurizing vats, it appears in the milk upon examination by the health department and casts doubt on the efficacy of the pasteurization process. It is not infectious.

*Pathogenic Action of Alpha-type Streptococci.*—Although constantly present in the normal mouth, usually without deleterious effects, these organisms can at times gain a foothold in the body. Hemolytic streptococci of the alpha type tend to cause chronic, but none the less dangerous, diseases. They are frequently found in abscessed teeth and diseased tonsils and are always present in saliva. From teeth and tonsils they may be carried to the joints and produce rheumatic conditions. They can also infect the heart valves causing a highly fatal heart disease, *bacterial endocarditis*. It is often because of the danger from alpha-type streptococci that infected teeth and tonsils are removed. This practice is falling into disuse.

**Lactic Streptococci.**—*Streptococcus Lactis.*—A second subdivision of streptococci is made up of varieties related to *Str. lactis*, the common milk-souring streptococcus, useful in cheese-making, and of which there are several subvarieties (or subspecies, depending on one's definition of a species). These are usually of the gamma type in blood agar but some may produce green zones. *Str. lactis* is always present in market milk, even of the best quality. It occurs in cow dung and its entrance into the milk is easily explained. It is quite harmless to man.

It occurs chiefly in short chains or pairs and the cells tend toward an oval shape. It grows rapidly in the milk at summer and body temperatures, usually overgrowing and suppressing the development of other organisms, some of which might otherwise cause the milk to putrefy. It ferments the lactose, producing lactic acid and the not unpleasant flavor characteristic of "cottage cheese." "Cottage cheese" is simply the casein of milk coagulated and flavored by the lactic acid and certain other aromatic products of the fermentation of lactose, such as diacetyl. *Str. lactis* has no ability to hydrolyze proteins like casein or gelatin.

*Streptococci Related to Streptococcus lactis.*—There are numerous closely related streptococci, some of them forming gas during the fermentation of lactose, and all of them forming lactic acid. One of these is *Str. cremoris*. Pure cultures of this organism called "starters," are used in making butter and cheese, the object being

to ensure that the milk or cream is soured by an organism which yields a pleasant flavor.\* *Str. lactis* may be termed the "type species" of the "lactic group." These cocci will grow well at temperatures around 10° C. but not over 35° C.

**The Enterococci.**—The third subdivision of streptococci, designated as enterococci, comprises a number of species of alpha, beta and gamma types of streptococci with wide tolerance of heat and cold and other influences unfavorable to other streptococci. The enterococci are commonly found in the intestinal tract and are represented by *Str. faecalis*. This organism often produces alpha-type zones of hemolysis in blood-agar plates, but some closely related species produce no change in blood agar (gamma type).

Related varieties are *Str. liquefaciens* and the so-called *Str. zymogenes*, which differs from the preceding only in being slightly hemolytic, producing zones of the beta type in blood agar. *Str. zymogenes* possesses an antigen which relates it to other beta hemolytic streptococci and it is found appended to Lancefield's system in group D. Many regard these streptococci as variants of *Str. faecalis*. However, both are proteolytic while *Str. faecalis* is not. They are also closely related, both in properties and habitat, to the lactic group, but they can be differentiated as shown in Table X. *Str. liquefaciens* and *Str. zymo-*



Fig. 268.—*Streptococcus liquefaciens* (× 900). (Frobisher and Denny, Jour. of Bact., Vol. 16, 1928.)

*genes* have several properties similar to *Str. lactis* but give rise to aromatic and bitter flavors in cheese. They occur in cow dung and have been found in sewage and in certain pathological conditions, although their pathogenic action is doubted.

All of the enterococci have a tendency to produce short chains and pairs of plump, ovoid cocci, and are commonly found in clumps suggestive of staphylococci (see Fig. 268).

\* Since rationing of butter, these flavoring substances have been taken from soured skim milk and added to various margarines along with vitamins to produce fairly acceptable (?) butter substitutes.

**Genus *Leuconostoc*.**—The student who recalls his elementary biology will remember a variety of Cyanophyceae, or blue-green algae, called *Nostoc*. It is characterized by its spherical cells occurring in long chains, strongly reminiscent of streptococci but of course much greater in diameter. The name *Leuconostoc* brings this organism to mind, inasmuch as *Leuconostoc* produces spherical cells in chains. Like the blue-green algae, also, the cells secrete thick zooglycal masses about themselves. Their size, however, is of the same order as that of the other true streptococci and there is no really close relationship to the algae. *Leuconostoc* contains no chlorophyll and is a typical, chemosynthetic schizomycete. It differs from other streptococci in forming carbon dioxide from dextrose.

*Leuconostoc mesenteroides*, the type species of the genus, grows chiefly in sugar refineries and mills, in the vats and drain pipes, metabolizing sugar. The organisms often become great nuisances, their gummy masses interfering with drainage and mixing.

The cells are spherical, measuring about 2 microns in diameter. They are enclosed in a homogeneous, gum-like substance somewhat like a large tough capsule. Short chains are formed which grow in compact masses adherent to solid surfaces. These masses become very large and form gummy excrescences called, by the French, "gomme de sucrerie." The gum is serologically related to the capsular carbohydrate of type II pneumococci.<sup>23</sup>

*L. citrovorum* and *L. dextranicus* are well known and are found in dairy products, especially butter starters (see the next chapter).

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## CHAPTER 35

### BACTERIA IN MILK

IN THE PRECEDING chapter we have seen the importance in milk of streptococci like *Str. lactis* and of beta hemolytic streptococci of the Lancefield groups A and B. Here we may further discuss the presence of these and other species of bacteria, previously described, in milk, and indicate their sanitary and other significances.

Milk secreted into the udder of healthy cows is sterile. It has a pH of around 6.8. Some bacteria are able to grow up into the milk



Fig. 269.—Sanitary milk production. Note the cleanliness of the cows, the absence of dung and straw, the good lighting and ventilation and the milking machines. (Courtesy of the DeLaval Separator Company.)

duct of the teat so that the first milk drawn (this should be discarded) usually contains numbers of them. These may include staphylococci, micrococci and several saprophytic types such as *Bacillus*, *Serratia*, etc., from soil. Except in cases where extra precautions are taken at the time of milking (see Figs. 269, 270 and 271), the milk receives additional contamination with various kinds of bacteria from the pail and other dairy utensils, from soil and dust in the air, from the flanks, tail and udder of the cow, and from the hands of milkers (Fig. 272).

**Numbers and Significance of Bacteria Normally in Milk.**—Ordinary market milk always contains strains of *Streptococcus*

*lactis*, *Escherichia* and *Aerobacter*, *Staphylococcus*, *Micrococcus*, *Actinomyces*, *Lactobacillus*, spore-formers, yeasts and molds, derived from the intestinal tract of the cows, from barnyard dust, utensils or hands. The presence of these usually *nonpathogenic* bacteria in milk is a serious matter only when they cause the milk to sour, putrefy, or develop undesirable flavors or conditions like "blue milk" or "red milk" or "ropiness" (see page 441), or when, because of their numbers, they show the milk to be dirty.

Since milk is an excellent medium for bacterial growth, the number of bacteria in it will increase steadily the longer it stands,

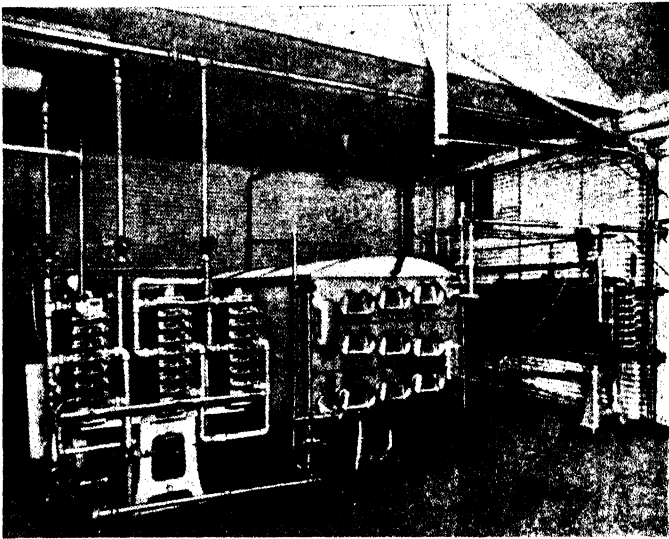


Fig. 270.—A small pasteurization plant. All of the pipes and tanks can be dismantled in a few minutes for cleaning with hot water. (George W. Putnam.)

especially if not refrigerated. Unpasteurized milk "sours" (coagulation at pH 4.8), due principally to the rapid growth of *Str. lactis*. Pasteurized milk usually does not sour so promptly because the streptococci are largely destroyed by the heat. It may putrefy because of the growth of spore-formers and other heat-resistant forms which do not ferment lactose. Among these may be micrococci and species of *Proteus* and *Bacillus*. Yeasts and molds may predominate later on. Many of these are very aciduric.

Stale or dirty milk, therefore, may contain millions of bacteria per cubic centimeter. The very best quality, fresh, certified "baby

milk" may contain from a few hundred to about three thousand, while good, general market milk may contain ten to fifty thousand organisms per cubic centimeter.

Obviously, in order to produce milk that is highly sanitary and has good "keeping" qualities, it is necessary to (a) have clean cows which are inspected periodically by competent veterinarians

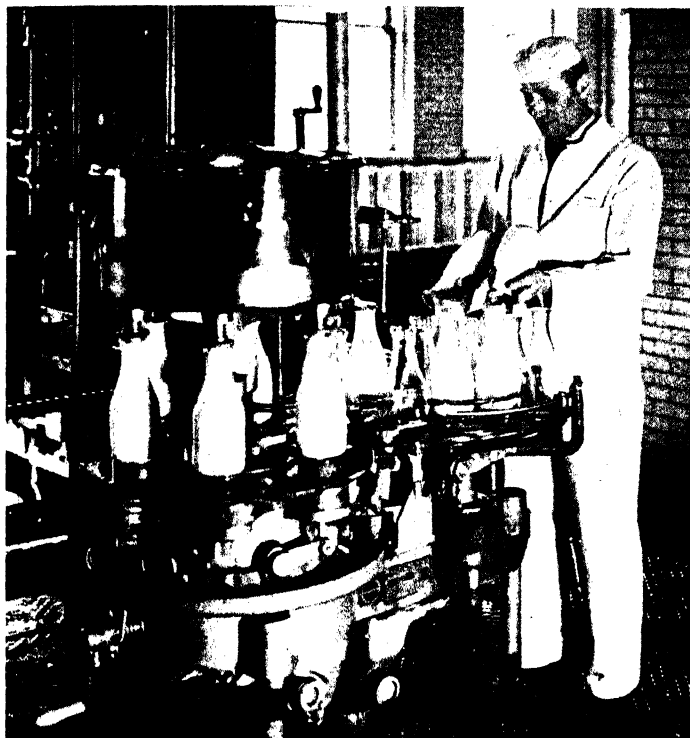


Fig. 271.—Sanitary bottling of milk. Compare with conditions shown in Fig. 272. (Courtesy of Cherry-Burrell Corporation, Chicago.)

to be sure that they are free from disease; (b) have all utensils absolutely clean and as nearly sterile as possible (plenty of very hot water). The use of paper milk bottles has much to recommend it. Studies by Tanner and others have shown the sanitary advantages.<sup>15</sup> (c) Insist on cleanliness of barns, clothing and person of milkers; (d) have all workers in and around the dairy examined medically each month or so.<sup>1, 2, 3</sup>

**Significance of Coli-like Organisms in Milk.**—*E. coli* and the genus *Aerobacter* are always present in market milk before pasteurization. However, these organisms are killed if the process is properly carried out. It is possible to determine this by making counts to determine if any *E. coli*, etc. have survived the heating. Samples of the processed milk are diluted and “plated out” by methods previously described (see page 240), but a medium is used which is selective for *E. coli*, and its allies the *Aerobacter*. The medium generally used is desoxycholate agar (see chapter on enteric organisms, page 488). Red colonies should be subcultured and studied



Fig. 272.—Unclean bottling of unpasteurized milk. (Photo by Lewis Hine. Courtesy of Cleanliness Institute.)

to prove their identity, and, if considerable numbers prove to be *E. coli* or *Aerobacter*, it is evident that the milk has not been properly pasteurized or that it has been contaminated by unclean conditions afterward. Another method of detecting improper pasteurization or handling of milk is the phosphatase test.

**The Phosphatase Test.\***—This test is based on the power of the

\* The Phosphatase Test. *Rapid Method for Use in the Field.* (Scharer, 1938)<sup>4, 5</sup>  
**Reagents**

A. 2,6-Dibromo-quinone-chloroimide (referred to below as BQC) dissolved in 95% ethyl alcohol and kept in tightly stoppered dark bottle. Remains stable for several days. Use with dropper delivering 50 drops per ml.

B. Buffered substrate contains disodium phenyl phosphate, sodium borate

heat-sensitive enzyme, phosphatase, normally present in milk, to liberate phenol from phosphoric-phenyl esters. When milk is heated, this enzyme becomes progressively inactivated; when heated at 143° F. for thirty minutes (pasteurization), 96 percent of the enzyme is destroyed, and heating above 145° F. for thirty minutes insures complete inactivation. When the milk has been under-heated (in respect to either temperature or time) or when there is an admixture of raw milk, the enzyme will be present in larger amounts than when the milk was properly processed. Quantitative determination reveals the degree of faulty pasteurization or subsequent contamination with raw milk. Over all ranges of temperature and times, *Mycobacterium tuberculosis* (the most resistant of the nonspore-forming pathogens commonly found in milk) is destroyed more quickly than phosphatase, so that a heat treatment adequate to inactivate the enzyme likewise kills this organism and all other common pathogenic bacteria. A sample of milk that does not have more phosphatase present than the standard allows can be regarded as safely pasteurized and free from subsequent contamination with raw milk.

This test will detect 0.5 percent raw milk mixed with pasteurized milk, or one degree below standard temperature, or five minutes' underheating during pasteurization. A color greater than that in a standard with 0.01 mg. phenol per cc. indicates improper handling of the milk.

Certain bacteria, as *Lactobacillus enzymothermophilus*, discovered and identified by Buck and further reported by Kaplan, both of the Baltimore City Health Department, will give falsely positive results in properly pasteurized milk because they produce a heat-stable phosphatase.<sup>6, 7</sup>

**Counting the Bacteria in Milk.**—In order to have some measure of the conditions under which milk has been produced and handled

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buffer, and magnesium. Dissolve in distilled water. Remove traces of free phenol by treatment with BQC and extraction with butyl alcohol. Substrate unstable and should be stored under refrigeration.

(A and B may be purchased ready prepared, or see ref. above.)

**Method**

1. Add 5 cc. buffered substrate to 0.5 cc. milk sample. Shake briefly.
  2. Incubate 10 minutes in water bath at 37° C.
  3. Remove and add 6 drops BQC solution. Shake well.
  4. After 5 minutes, compare color with opaque standards (see ref. above).
- Properly pasteurized milk will be gray or brown.  
Properly pasteurized cream will be gray or white.  
Raw milk or cream will be an intense blue. Intensity of color is proportional to degree of underpasteurization or amount of added raw milk.

and to have a legal control over its sanitary quality, health departments and dealers have set up various standards by which to judge milk.<sup>1, 2, 3, 8</sup> Important among these standards is the number of bacteria present in the milk. The sample must be collected in *sterile bottles*, must represent the *whole supply* and must be *iced* and *examined promptly*. For microscopic counts it may be preserved with formaldehyde.

**Breed Count.**—The number of bacteria in market milk is commonly estimated in health departments by one or both of two methods. In one (the Breed count), 0.01 cc. of the milk is evenly smeared on a glass slide over an area of exactly one square centimeter. This smear is treated with xylol and alcohol to remove fat

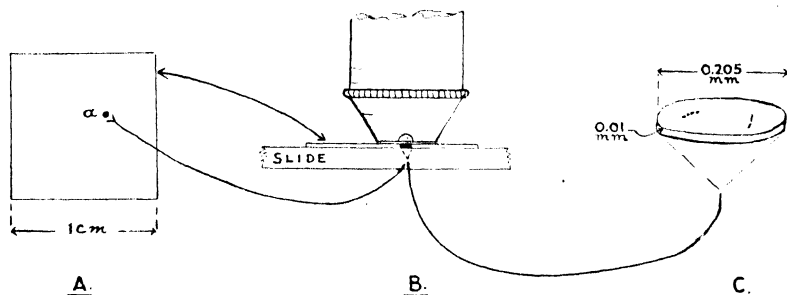


Fig. 273.—The direct microscopic examination of milk.

A, Area 1 cm. square over which 0.01 cc. of milk is spread to prepare the smear. Small area (a) represents portion seen in one objective field.

B, Oil immersion objective focussed on milk film, showing area observed.

C, Diagram representing magnified disc of milk subtended by the microscope objective. This disc contains the bacteria in approximately 1/300,000 cc. of milk.

and is stained with methylene blue.\* A microscope, standardized by means of an object micrometer so that the field covers an area of approximately 1/3000 of a square centimeter (field diameter 0.205 mm.), is then used to count the visible bacteria. Using the data concerning the area covered and the volume of milk used, the

**\* Newman's stain for milk smears:**

Methylene blue (certified) .....	2.0 gm.
Ethyl alcohol (95 percent) .....	60.0 cc.
Xylene, C.P. ....	40.0 cc.
Acetic acid, glacial .....	6.0 cc.

Dissolve the dye in warm alcohol. Add xylene and acid and filter. Keep in stoppered bottle.

1. Immerse smear about 1 minute.
2. Remove and drain until dry.
3. Wash thoroughly in water.

total number of bacteria per cubic centimeter of milk may be computed (Fig. 273). In addition, mastitis may be detected by the observation of large numbers of pus cells and streptococci. This method is coming to be used more as a rough preliminary assay of the approximate quality of milk than as an accurate counting procedure. It is quick and inexpensive. (Compare with microscopic examination of soil, see page 405.)

**Plate Count.**—In the second method, 1 cc. of various dilutions (1/10, 1/100, 1/1000, etc.) of the sample are placed in sterile Petri dishes. Each dish then receives 12 to 15 cc. of melted agar of a standard composition,\* cooled to about 45° C., and the dish is rotated to mix the agar and diluted milk. After 48 hours' incubation at 37° C. colonies of bacteria will have developed which are visible to the naked eye or by the use of a 2× reading glass. Each of these colonies develops, theoretically, from a single organism. The number of colonies in any plate multiplied by the dilution of the milk in that plate, therefore, gives an approximation of the number of bacteria per cubic centimeter of the milk. Of course, many bacteria fail to grow at 37° C. or in such media. Considerable variation in results may be expected if other media or temperatures of incubation are used.<sup>9</sup> The presence of certain heat-resistant streptococci, lactobacilli, etc., gives high plate counts even in pasteurized milk.<sup>6, 11</sup>

**Numerical Relationship Between Breed and Plate Counts.**—The Breed count is usually five to ten or more times as high as the plate count because the former counts *individuals*, even those in clumps, and also *dead* bacteria, while the latter counts only live bacteria capable of developing in the medium used at 37° C., *the clumps counting only as single bacteria*. Each clump forms *one* colony. Sometimes the clumps contain several dozen individual cells, yet they always yield only one colony. The conditions under which these tests should be done are prescribed in a manual called "Standard Methods for the Examination of Dairy Products."<sup>4, 5</sup>

**Grades of Milk.**—The actual numbers of bacteria permissible

\* Agar for milk counts:

Water . . . . .	1000 cc.
Meat extract . . . . .	3 gm.
Tryptone . . . . .	5 gm.
Dextrose . . . . .	1 gm.
Skimmed milk . . . . .	10 cc.*
Agar . . . . .	15 gm.
pH 7.0	

\* For milk dilutions greater than 1/10.



in milks of various grades vary in different cities. A good guide is the standard ordinance of the U. S. Public Health Service.<sup>1</sup> New York City has somewhat different standards.<sup>10</sup>

Abstract of milk grades from Pub. Health Bulletin No. 220 Milk Ordinance and Code of the U. S. P. H. S.

#### *Grade A Raw Milk*

Grade A raw milk is raw milk the average bacterial plate count of which does not exceed 50,000 per cubic centimeter, or the average direct microscopic count of which does not exceed 50,000 per cubic centimeter if clumps are counted or 200,000 per cubic centimeter if individual organisms are counted, or the average reduction time of which is not less than 8 hours: Provided, That if it is to be pasteurized the corresponding limits shall be 200,000 per cubic centimeter, 800,000 per cubic centimeter, and 6 hours, respectively; and which is produced upon dairy farms conforming with all of the prescribed items of sanitation.

#### *Grade A Pasteurized Milk*

Grade A pasteurized milk is grade A raw milk, with such exceptions as are indicated if the milk is to be pasteurized, which has been pasteurized, cooled, and bottled in a milk plant conforming with all of the prescribed items of sanitation and the average bacterial plate count of which at no time after pasteurization and until delivery exceeds 30,000 per cubic centimeter.

The grading of a pasteurized-milk supply shall include the inspection of receiving and collecting stations.

#### *Grade B Raw Milk*

Grade B raw milk is raw milk which violates the bacterial standard and/or the abortion testing requirement for grade A raw milk, but which conforms with all other requirements for grade A raw milk, and has an average bacterial plate count not exceeding 1,000,000 per cubic centimeter, or an average direct microscopic count not exceeding 1,000,000 per cubic centimeter if clumps are counted or 4,000,000 per cubic centimeter if individual organisms are counted, or an average reduction time of not less than 3½ hours.

#### *Grade B Pasteurized Milk*

Grade B pasteurized milk is pasteurized milk which violates the bacterial standard for grade A pasteurized milk and/or the provision of lip-cover caps and/or the requirement that grade A raw milk be used, but which conforms with all other requirements for grade A pasteurized milk, has been made from raw milk of not less than grade B quality, and has an average bacterial plate count after pasteurization and before delivery not exceeding 50,000 per cubic centimeter.

**The Reductase Test.**—In the section on systematic studies of bacteria it was explained that actively growing bacteria bring about a lowered oxidation-reduction potential in their medium which can be detected by the use of methylene blue, for with a lowering of the O-R potential this dye changes in color from blue to white. The test is used principally on raw milk and furnishes a rough approximation of its bacteriological quality. In performing

the test 10 cc. of milk sample are pipetted into a sterile tube and 1 cc. of a standard methylene blue solution (final concentration 1:300,000) is added. The tube is closed with a rubber stopper and inverted once to mix. It is placed at 37° C. in the water bath immediately.

After 5 minutes, the tube is inverted once to promote uniform creaming, and is not disturbed thereafter. Observations are made at 5 minutes, 20 minutes, 30 minutes, 1 hour, and 1½ hours.

The "methylene blue reduction time" is the interval between the placing of the tubes in the 37° C. water bath and the disappearance of the blue color from the milk. It is customary to consider reduction complete when four-fifths of the contents of the tube have turned white. Sometimes the reduction is irregular or mottled in distribution.

**Certified Milk.**—If milk is to be offered for sale unpasteurized, it is often required that it be produced only under very carefully supervised conditions. The *American Association of Medical Milk Commissions* has established rules and regulations concerning veterinary inspection of cows, sanitation of barns, utensils, etc., which may be used by health departments and milk dealers in certifying qualified farms to produce such milk.<sup>12</sup> It is usually called *Certified Milk* or "baby milk." The use of certified milk has much to recommend it, especially its cleanliness. It is said also to contain a larger proportion of certain vitamins essential for infants than milk which has been heated. This is a disputed point.

Most cities and states as well as the *A.A.M.M.C.* require that all persons occupied in preparing certified milk, or, indeed, any food for the public, whether it be certified milk or not, be examined periodically for typhoid, paratyphoid and dysentery bacilli. Examinations for *Corynebacterium diphtheriae*, tuberculosis, scarlet fever and other diseases are also required for certified milk handlers.

**Lactobacilli in Milk.**—The organisms of this genus are widely distributed in soil, on plants and around barnyards, and are as frequently present in market milk as *Str. lactis*, although possibly not in such large numbers. They are important in the dairy industry in several ways. Lactobacilli, as their name implies, are associated with lactic acid production. They are highly fermentative organisms, forming large amounts of acid, especially lactic acid, and have the ability to endure higher degrees of acidity than many other species of bacteria. They are therefore described as being *aciduric*, although the name *acidophilic* or "acid-loving" is

widely used to describe them. They are not actively proteolytic. Morphologically, they are slender, pleomorphic rods (Fig. 274), often assuming granular forms closely resembling *Streptococcus lactis* or organisms of the genus *Corynebacterium*, a group of club-shaped organisms to which the bacillus of diphtheria belongs. Like the streptococci and the corynebacteria, they are gram-positive. They are nonmotile, nonspore-forming and anaerobic or micro-aerophilic. (The term *micro-aerophilic* indicates that the organisms are not strictly anaerobic but prefer an atmosphere in which free oxygen is somewhat deficient.) Several species are heat-loving (thermophilic) and at least one of the 15 listed species of lacto-



Fig. 274.—*Lactobacillus bulgaricus* ( $\times 600$ ).

bacilli (*L. thermophilus*) grows well at temperatures as high as  $62^{\circ}\text{C}$ ., a temperature quickly fatal to many nonspore-forming rods, while several other species (*L. caucasicus*, *L. acidophilus*) can grow at temperatures around  $45^{\circ}\text{C}$ .<sup>11</sup> A good deal of difference of opinion exists as to the proper systematic position of these organisms.

While commonly found in milk and cheese (they are important in the ripening of cheddar cheese), certain species are also found in large numbers in the intestinal contents of infants (*L. bifidus*) and in the vagina (*L. acidophilus*), while others occur in souring vegetable products like ensilage and sauerkraut (see chapter on industrial aspects, page 593).

**Fermented Milk Beverages.**—In certain countries lactobacilli have been used for hundreds of years in combination with certain yeasts and streptococci, to produce beverages of fermented milk. The *yoghurt* (*L. bulgaricus*) of central Europe, the *busa* of Turkestan, the *kefir* (*L. caucasicus*) of the Cossacks, the *koumiss* of Russia and the *leben* of Egypt are examples of these. In the old days, of course, the bacterial nature of these processes was unknown.

The lactobacilli act in company with the other bacteria of milk. *Kefir*, made from mare's milk, is prepared by putting "kefir grains" (small, cauliflower-like masses) into the milk. These grains consist of dried masses of lactobacilli, yeasts and streptococci (*S. lactis*) and probably other organisms. Their combined growth



Fig. 275.—Elie Metchnikoff (1845–1916). (From Garrison, "History of Medicine.")

yields a characteristically flavored, soured milk. The *kefir* grains are found in the bottom of the vessels of fermented milk.

In this country, *Lactobacillus acidophilus* is used to produce a similar fermented milk product called "acidophilus milk." It is obtainable from some of the larger dairies and pharmaceutical houses.

**Health Value of Fermented Milk.**—Metchnikoff (Fig. 275) observed that, among peoples drinking these sour milk beverages, longevity was common. He attributed this to the beneficial effects of the soured milk, and particularly to *Lactobacillus bulgaricus* or the "Bulgarian bacillus" found in *yoghurt* and *leben*. He became the foremost exponent of the idea that if these fermentative organisms could be implanted in the intestine, life might be pro-

longed. This is probably erroneous. However, putrefactive processes related to various disturbances like chronic constipation which result in "auto-intoxication" (the latter probably due to the absorption of poisons such as hydrogen sulfide, amines, mercaptans and various organic acids, alcohols, etc., derived from the action of putrefactive bacteria on the contents of the intestine), are overcome by the high acidity produced by the fermentative activity of certain lactobacilli in the intestine. (We have already seen that acidity is unfavorable to the growth of many bacteria.) This view is further supported by the observation that the intestinal flora of healthy, nursing infants is composed chiefly of lactobacilli (*L. bifidus*). Attempts to implant *L. bulgaricus* in the intestine, however, met with failure.

It was later found that *Lactobacillus bulgaricus* cannot grow in fluids having low surface tensions, while *L. acidophilus*, a closely related species, can. Bile produces low surface tension and it may be due to this that *L. bulgaricus* cannot be implanted in the intestine, since the intestinal contents contain bile. It was found possible to secure a considerable growth of *L. acidophilus* in the lower bowel, provided the patient consumed quantities of lactose (milk sugar) in order to provide a source of energy for the organisms. The beneficial effects of such implantations are doubted by some authorities today.

Certain lactobacilli are said to be responsible for dental decay because of the acid they produce in crevices in and around teeth. Parsons has shown that aciduric streptococci may be of equal importance in this respect.<sup>16</sup> Acidity of the secretion of the vagina may be due largely to the growth in the vaginal secretions of organisms formerly called Döderlein's bacillus but now thought to be identical with *L. acidophilus*.

**Genus *Propionibacterium*.**—These are gram-positive, anaerobic, facultative or micro-aerophilic, nonmotile, nonspore-forming bacilli. They are very pleomorphic and may be closely related to the lactobacilli. They occur chiefly in milk and its products, such as cheese. They are quite active in attacking carbohydrates and metabolize many organic acids and alcohols and form considerable amounts of acid, chiefly propionic, whence their name. They also form considerable amounts of carbon dioxide gas. They are of importance in producing the flavors and the "eyes" or holes in Swiss (Emmenthal) cheese. The type species is *P. freudenreichii*.

**Transmission of Diseases by Milk.**—The infectious diseases most commonly transmitted by milk are typhoid fever, scarlet

fever and septic sore throat, dysentery, diphtheria, tuberculosis and undulant fever.

The human intestinal infections, dysentery and typhoid fever, when transmitted by milk, practically always represent contamination of the milk by a carrier of these organisms, since cattle are not subject to these diseases. The milk must indeed be handled by very unclean people for it to become contaminated in the first place. If it is not refrigerated properly, or is held at warm summer temperatures, the initial infection may increase enormously by growth of the disease organisms in it, especially if the normal milk organisms do not produce a prompt souring, which is unfavorable to most of the pathogens. The same circumstances which favor transmission of intestinal infections by milk probably favor transmission of diphtheria. That is, infection is probably introduced after the milk is drawn, by a carrier. Subsequent improper storage temperatures may cause multiplication of the diphtheria bacilli if the normal flora does not overgrow them. Possibly the bovine udder may become infected with *C. diphtheriae*, but the evidence for this as an important cause of diphtheria is not very direct.

If the internal structures of the cow's udder be diseased, other pathogenic bacteria may be present in the milk in enormous numbers even as it is secreted. The organisms causing several diseases in human beings can also infect cattle and often localize in the udder. Tuberculosis, scarlet fever, septic sore throat,<sup>13</sup> and undulant fever sometimes result from such udder infections. Bovine tuberculosis and undulant fever are easily transmitted from animal to animal. The number and variety of organisms in milk depend upon the cleanliness of the conditions surrounding the milking process and on the *health of the cow* and of *dairy workers*.<sup>14</sup>

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## SOME INDUSTRIAL APPLICATIONS OF BACTERIOLOGY

For many years it has been known that microorganisms play an important rôle in the manufacture of a number of substances used commercially. Some of these biological processes have been very extensively investigated by expert bacteriologists, and in

\* Flint, from Smyth and Obold, "Industrial Bacteriology," Williams and Wilkins

a number of processes certain specific organisms have been discovered as the active and essential agents. This is particularly true in the manufacture of commercial solvents (alcohols of various kinds, acetic and other organic acids, acetone and glycerin). In a number of processes, however, final data on the exact rôle of definite species of bacteria have not yet been obtained. The fermentation of coffee and cocoa are examples of the empirical type of bacteriology in industry. The field of industrial bacteriology is an open one and offers excellent opportunities for the bacteriologist.

**Factors of Importance in Developing Industrial Processes.**—In arranging or studying an industrial process based upon the action of bacteria, a great many details must be given consideration.<sup>1</sup> Some of the more important of these may be grouped, roughly, in four categories.

**1. Purity of Cultures.**—It must be ascertained whether absolutely *pure* cultures *must* be used, or whether the mere *predominance* of one organism is sufficient. This may be a deciding factor, as the cost of preparing and maintaining pure cultures throughout a process may be excessive. For purposes of discussion let us assume a pure culture to be requisite.

The organism must be able to grow well in the medium to be used and under the conditions of the process. This will entail very exact studies of *optimum* conditions of aerobiosis, temperature and pH, and an appropriate adjustment of the process and apparatus to provide those conditions. The organism must be one which will evolve the desired end-products or produce the desired result in the medium furnished, in amounts or degree sufficient to yield a profit. Some firms have “pet” strains of bacteria for producing certain products, such as butyl alcohol, which they have “developed” (selected variants) for these purposes.

**2. Medium or Raw Material.**—The second factor to be considered is the substrate or medium. It should support luxuriant growth of the organism to be used. Further, it must be available constantly and at costs compatible with profit. Expensive handling machinery may be needed for some substrates.

An important item is the possible necessity of a preliminary treatment such as liming of very acid yeast “slops,” distillery wastes, molasses and whey. Some substrates, such as sawdust or fiber, may need preliminary “digestion” with hot acid or alkali to hydrolyze them to fermentable substances. This all adds to the expense and time.



**3. Nature of the Process.**—A third consideration, the details of which arise from the requirements of the first two, is the process itself. It is obvious enough that the more complicated and exacting the system of cultural details and preliminary heatings, dilutings and digestions, as well as the type of machinery (“cracking” stills, tanks, pumps) to handle the end- and by-products and the final wastes, the greater the cost and therefore the less the commercial practicability of any process. Any time-consuming “aging” or “ripening” processes eat into the credit side of the ledger. Sometimes very desirable end- or by-products may be found in commercial fermentations, yet the cost of their recovery may be prohibitive.

**4. Preliminary Experimentation.**—The fourth matter to be considered in industrial bacteriology is preliminary trial and investigation. The bacteriologist working with 10 cc. test-tube cultures may find many valuable things. When attempts are made to reproduce the test-tube experiments on a 100,000-gallon factory scale, however, the laboratory discoveries often fail to yield the promised result. Any process developed in the experimental laboratory must next prove its worth in the factory. All may depend on such a seemingly far-removed detail as international relations. These may affect the cost of importation of some raw product essential to the process under investigation. Then the industrialist turns to home resources, goes to Washington with a carpet bag, or employs a resourceful bacteriologist!

The whole matter is a complex of bacteriology, chemistry, engineering and economics and cannot be more fully discussed here. Many of the chemical and bacteriological processes in use at present are patented and secret, and specific strains of bacteria, yeasts and molds, which are zealously guarded, are often carefully developed in the laboratories of manufacturing concerns. We shall, therefore, present only general principles and point out the possibilities for research and advancement in industrial bacteriology, leaving the student, if he is interested, to consult original papers for greater detail. The commercial production of penicillin has already been discussed (see page 132).

In commerce fermentation, oxidation, proteolysis, lipolysis, synthesis and other activities of yeasts, molds and bacteria, are employed. The products differ, depending upon the kinds of micro-organisms involved and the substance which they are required to alter or grow upon, and the conditions of growth during the process. One industry of very ancient origin, but in which modern bacteri-

ology has come to play an important part, is cheese manufacture. Both fermentation and proteolysis, as well as lipolysis, are important in this process.

**Cheese and Other Dairy Products.**—The use of members of the genus *Lactobacillus* and of *Streptococcus lactis*, as well as of yeasts, in preparing fermented milk beverages has been described (page 567). Pure cultures of *Lactobacillus* (*L. helveticus*) are also widely used as starters for various dairy products which depend on lactose fermentation. It has been pointed out also that some butter is made by churning cream which has been soured by means of cultures of *Str. lactis*, *Str. cremoris* and related organisms. These form pleasantly flavored volatile products during their fermentation of

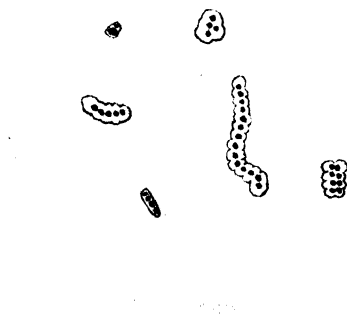
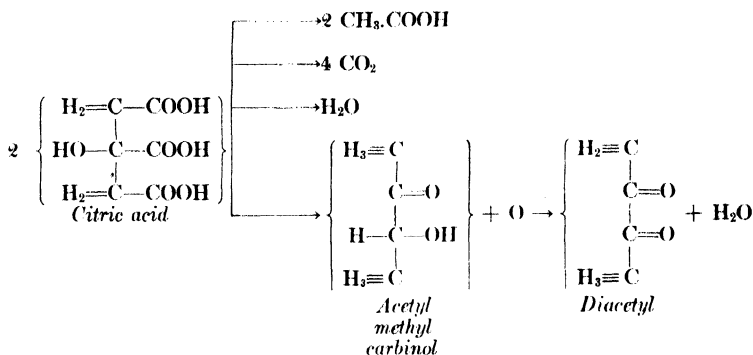


Fig. 276.—*Leuconostoc citrovorum* ( $\times 900$ ).

the lactose or milk sugar. Their rapid growth in the preliminary stages of butter-and-cheese-making produces an acidity which inhibits development of undesirable bacteria such as the coli-aerogenes group. Cultures of such organisms are obtainable commercially for the purpose of inducing favorable souring, and are known as "starters."

**Butter.**—*Streptococcus lactis* and *Str. cremoris*, commonly occurring in milk, are not alone responsible for the desirable flavors in butter. Closely related species capable of attacking citric acid under acid conditions, *Str. citrovorus* and *Str. paracitrovorus* (also classed as *Leuconostoc dextranicum* and *Leuconostoc citrovorum*, respectively) are of great importance (Fig. 276). The exact relationships of these species is not clear but it seems likely that

some *Leuconostoc* may represent encapsulated variants of *Str. citrovorus* and similar species. They produce diacetyl (see below)



which has a pleasant, "buttery" aroma. The addition of a little (0.1 percent) citric acid to cream or milk for butter aids in the production of these aromas, while the necessary acidity for satisfactory flavor production is produced by *Str. lactis*, *et al.* Commercial or home butter starters should contain these citric acid-fermenting strains in large numbers.

Sterilized milk is used as a medium for maintaining or shipping transfers from prepared mixtures of these cocci. Much experience is required to maintain the mixed cultures in such a condition that they will give the best results. Apparently the organisms vary considerably.

There are many molds and bacteria which cause spoilage of butter. Development of undesirable flavors in butter, especially rancidity, is due in great part to the formation of butyric acid as a result of lipolysis.<sup>2</sup>

*Cheese*.—The manufacture of cheese is one of the industries of antiquity. The bacteriology of the processes, however, is still not completely known but is understood to a degree sufficient for the desired types of cheese to be made.

Three general types of cheese may be mentioned, (a) soft, acid-curd or cottage type cheese and cream cheese (these are eaten in a fresh or unripened state); (b) hard- or rennet-curd cheese, including American, Roquefort, Cheddar, and Swiss or Emmenthal (these are "ripened" by the growth in them of bacteria or molds or both, which do not cause extensive proteolysis); and (c) soft or semisoft rennet-curd cheese, of which Camembert, Limburger and Liederkranz are types (these are ripened by more or less

proteolytic organisms which soften the curd). The hardness of cheese depends to some extent on moisture and fat content as well as on heating and acidity of the curd. There is some disagreement as to the classification of cheeses. A list of cheeses is given in Table XII.

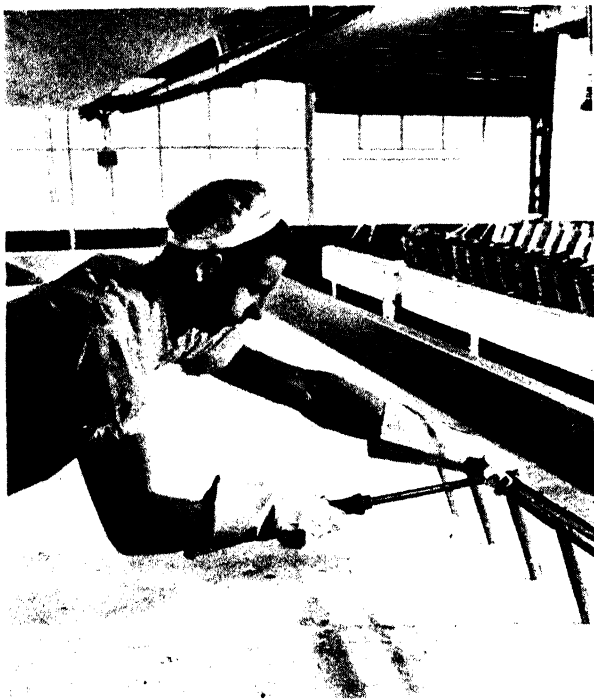


Fig. 277.—Liquid milk becomes “curd” and “whey.” After “starter” and rennet are added, the milk forms into a soft curd much like “junket.” When it has reached the correct firmness, it is cut into small cubes ( $\frac{1}{4}$  inch) by special wire knives. The whey is slowly expelled from these cubes as the curd and whey are heated, while agitated by revolving paddles. The picture shows the cutting operation. (Courtesy of Kraft-Phenix Cheese Corporation.)

*Soft, Acid-curd Cheese.*—In cheeses of this type, the bacteria normally present in the milk are allowed to ferment the lactose, the lactic acid which is thus formed coagulating the casein which is then strained out, drained and sold. Rennet may be added to hasten the coagulation and make the curd firmer. Salt may be added to taste after draining the curd. *Streptococcus lactis* is the

predominating organism, although others may be added in pure culture as starters. Certain yeastlike plants (*Oidium lactis*) also aid. Proteolytic organisms like *Str. liquefaciens* are responsible for undesirable bitter flavors and early spoilage. It is important to use fresh, clean milk to avoid gas production by *Aerobacter*, etc., and anaerobes, and putrefaction by *Pseudomonas*, *Bacillus*, etc. Hot pressed flavored cream may be made into cream cheese.<sup>3</sup>



Fig. 278.—The curd is milled, salted. When cheddaring is complete (judged by the firmness of the curd, its texture and acidity) the curd is milled (cut into small pieces) in preparation for salting and pressing. About  $1\frac{1}{2}$  pounds of salt are used to 100 pounds of curd. After salting, the curd is placed in cheesecloth or cellophane lined "hoops" of the desired size and shape for pressing. (Courtesy of Kraft-Phenix Cheese Corporation.)

*Hard-curd Cheese.*—In making hard- or rennet-curd cheeses, such as Cheddar cheese, the milk is first allowed to become slightly acid through normal fermentation or souring. Sometimes cultures of *Str. lactis* or *Str. paracitrovorus* are added to hasten this and improve flavor.<sup>4</sup> Rennet is then added to make an elastic, rubbery curd which is later chopped up (Figs. 277 and 278) into

pieces about 1 inch in diameter and heated. After heating to about 98° F. the curd becomes firmer and whey separates and is drained off and may be used for stock feed. The clumped masses of firm curd are chopped again, and piled up to press out whey. This is called "cheddaring" in Cheddar cheese making. The curd is again milled, salted, drained, and pressed in hoops to ripen. Ripening goes on at about 55° F.

During the process various bacteria and molds, the varieties depending on the kind of cheese. (*e.g.*, *Str. lactis*, *Str. cremoris*, lactobacilli, *Oidium lactis*, *Aspergillus* and *Penicillium*), are allowed to continue a slow fermentative and putrefactive action, the products of these processes yielding the substances responsible for the characteristic flavors, textures and aromas of various cheeses. Prominent among these are lactic, butyric and acetic acids, ammonia and various amines derived from the decomposition of the lactose, the fat and the proteins, as well as various esters such as those which give flavors to ripe fruit juices. In addition, as many of these organisms synthesize vitamins, especially nicotinic acid, and vitamins of the B complex, the nutritive value of the ripened cheeses is increased. The *Escherichia*, *Aerobacter* and *Clostridium* are undesirable since they produce gassy cheeses and "off" flavors; they may be especially active in the early stages. It has already been pointed out that the propionibacter are active in the ripening and flavor of Swiss cheese. The "eyes" in Swiss cheese are due to the production of carbon dioxide by the bacteria, while flavors are due in part to the formation of glycerol, propionic and succinic acids by these organisms.

TABLE XII

CHEESES<sup>5</sup>*Soft cheeses:*

Cottage	}	Not ripened
Cream		
Camembert	}	Ripened by molds
Brie		
Limburger	}	Ripened by bacteria
Liederkranz		

*Hard cheeses:*

Gorgonzola	}	Ripened by molds
Roquefort		
Emmenthal (Swiss)	}	Ripened by gas-forming bacteria (holes)
Parmesan		
Edam	}	Ripened by bacteria (no holes)
Cheddar		

Much depends on the way the cheese is made.<sup>6, 7, 7a, 7b, 7c</sup> Swiss cheese (Fig. 279) is heated to about 55° C. early in the process to prevent overgrowth of the streptococci and permit growth of the thermophilic lactobacilli (*L. thermophilus*, *L. lactis*, etc.) which give a certain desired texture and flavor. These subside after a time and *Propionibacterium* are then favored by incubation at lower temperature.

Roquefort cheese contains as high as 4 percent salt and relatively little moisture. This prevents growth of most bacteria, as does also the low ripening temperature of 9° C., although *Str.*

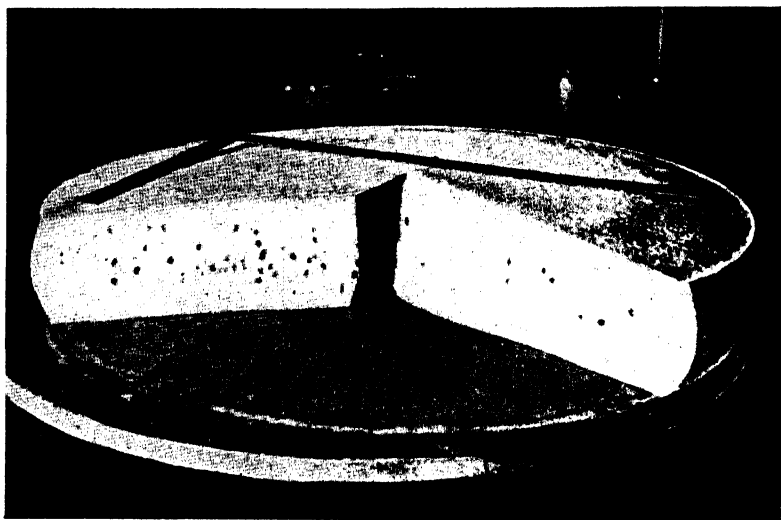


Fig. 279.—Swiss cheese, showing holes due to gas produced by the bacteria in the cheese before it hardened. (J. D. Frederiksen, "The Story of Milk," by permission of The Macmillan Company, publishers.)

*lactis* may continue in large numbers for several days after the initial souring. A species of mold (*Penicillium roqueforti*), however, is introduced by the admixture of spores with the curd before it is put into the hoops for ripening. These grow in the interior producing the "sharp" flavor so characteristic of this type of cheese (Fig. 281). As the mold is aerobic, perforations are made in the cheese to aerate the interior. The flavor results from the production of caproic, caprylic and capric acids from hydrolysis of the butter fat, as well as from the delectable volatile products of casein and lactose decomposition, some of which have been

mentioned above. The development of proper flavors depends in great part on suitable conditions of temperature and humidity of the atmosphere. In France the air in certain caves is especially favorable. In the United States air-conditioning of the ripening rooms is resorted to. (See Figs. 280 and 282.)



Fig. 280.—Cave in Roquefort, France, in which conditions are particularly favorable for the ripening of Roquefort cheese. See also Figure 282.



Fig. 281.—Roquefort cheese, showing the mold (*Penicillium roqueforti*) penetrating the interior of the mass. (Reprinted by permission from B. W. Hammer's "Dairy Bacteriology," John Wiley & Sons, Inc., publishers.)

In cheeses like Camembert the mold (*Penicillium camemberti*), being more strictly aerobic, grows on the surface, the liquefaction characteristic of this cheese proceeding from without inward. Probably *Oidium lactis* also plays an important rôle in the ripening (Fig. 282). Camembert cheese contains more water and less salt than Roquefort and is therefore softer to begin with.



**Vinegar and Acetic Acid. The *Acetobacter*.**—Acetic acid is almost entirely responsible for the sour taste of vinegar. Indeed, a slightly sweetened, 3 to 5 percent aqueous solution of chemically pure acetic acid makes a passable substitute for vinegar. Commercial vinegar contains 4 percent acid. The acid of naturally soured vinegar is derived from alcohol by the action of living organisms which are included in the family Acetobacteriaceae (genus *Acetobacter*). Pleasant flavors of natural vinegar are given by traces of various esters like ethyl acetate, and by alcohol, sugars, glycerin,



Fig. 282.—Camembert cheeses ripening in a room which is carefully “air-conditioned.” (Courtesy of Kraft-Phenix Cheese Corporation.)

etc., in small amounts. The organisms thrive on the surface of wine, “hard” cider, beer, or any nutrient liquor containing alcohol. Preliminary fermentation to produce the necessary alcohol is often carried out by means of *Saccharomyces cerevisiae* (brewers’ yeast). The *Acetobacter* later form a thick scum or pellicle, oxidizing the alcohol to acetic acid as a source of energy and utilizing other substances present as sources of food. They often ruin wine during the home manufacture of this beverage. The pellicle tends to become slimy with age (mucoid variation) and then constitutes the “mother of vinegar.”

Inasmuch as the change from alcohol to acetic acid is principally an aerobic oxidative process, it is evident that it will go on best when a large surface is exposed to the air for the growth of the *Acetobacter*, since they are strict aerobes. Therefore, in a commonly used commercial process, known as the "quick" or "generator" process for the production of acetic acid, alcoholic fluids are made

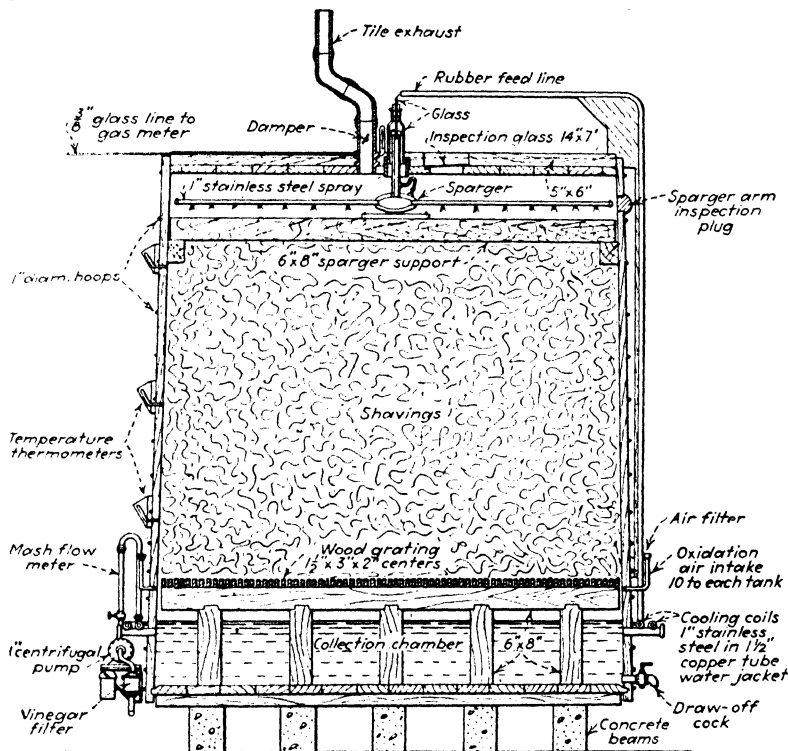


Fig. 283.— Cross section of the Frings generator. The alcoholic liquor is sprayed over the shavings by the rotating stainless steel spray near the top. Note the thermometers, cooling coils and air intakes. (Courtesy A. E. Hansen, Food Industries, Vol. 7.)

to trickle in a thin stream through a tall cylinder filled with shavings of beechwood, wicker-work, coke or tile, previously inoculated with *Acetobacter*. Air is made to circulate freely through the cylinder around the wet surfaces of the shavings. Heat is evolved and in large generators used in commercial work cooling devices must be resorted to. Due to the acidity of the product, wooden tanks and

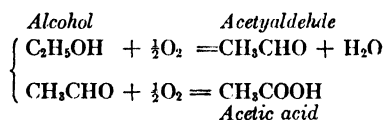
pipes are used. The acidity also eliminates much of the danger of contamination by extraneous organisms (Fig. 283).

The *Acetobacter* are rods about 0.5 microns by 8 microns, although species vary in size. Branching involution forms and large, swollen cells frequently occur (Fig. 284). Some species are motile. A species of historical interest is *Acetobacter (Mycoderma) aceti*, originally used by Pasteur to demonstrate the biological nature of vinegar formation. Species important in commercial vinegar pro-



Fig. 284.—*Acetobacter aceti*. Note the swollen involution forms ( $\times 300$ ).

duction are *A. schuezenbachii* and *A. orleanense*. In actual practice, several species of *Acetobacter* act jointly. The overall reactions probably are as follows:



*Home Vinegar Manufacture.*<sup>8</sup>—Recommendations for home vinegar manufacture illustrate the application of general principles to the slow or Orleans process. Apples are selected which are fully ripe, but sound and undecayed. Apples of high sugar content yield vinegar of high acid content, since it is the sugar which is eventually turned into acetic acid. *Red Astrachan*, *Tolman Sweet* and *Sweet Bough* are useful varieties. The cider is allowed to settle

for two or three days and is pumped or siphoned into barrels (clean and scalded to kill microorganisms producing undesirable fermentations) until they are about three-quarters full. The barrels are laid on their sides with the bung-holes open (but screened) and up. A cake of yeast or a pure culture of selected wine yeast is emulsified in half a cupful of water and added to each barrel. This promotes alcoholic fermentation, which is complete in from three to six months, depending on temperatures. In commerce temperatures are carefully controlled. At the end of this time, the clear alcoholic liquor is drawn off, the barrel cleaned, and the hard cider replaced. If the alcohol content is more than about 10 percent, the fluid must be suitably diluted, as too much alcohol is unfavorable to *Acetobacter*. A small amount of good quality natural vinegar or lactic acid is added to adjust the acid content so that it is unfavorable to undesirable bacteria. This may require 5 percent by volume, or an acidity around 2 percent. The use of vinegar also accomplishes inoculation. As the oxidation of alcohol to vinegar is strictly aerobic, the bung-hole must be left open. A cotton plug is useful to keep out extraneous organisms. Up to this stage, the barrels have been filled only three-quarters full, in order to allow a broad surface for the absorption of oxygen. But when acidification has reached completion, the barrels are filled, the bungs driven in and the product stored in a cool place to preserve the vinegar. Exclusion of oxygen is requisite now to prevent further oxidation of the acetic acid to carbon dioxide and water by yeasts. In commerce, pasteurization is used to stabilize the vinegar if it is not to be used at once.

**Alcohol Manufacture.**—Commercially, alcohol is derived from the fermentation of various carbohydrates, chiefly by yeasts. In Germany potatoes are widely used as the source of carbohydrate. French manufacturers use sugar beets. Starches and cellulose which may be hydrolyzed by means of enzymes or heat and acid to simpler carbohydrates which yeasts can ferment, may be used. Corncobs and sawdust may be so treated. Probably the most widely used source of industrial alcohol in the United States is crude molasses. It is easily handled in pumps and tanks and is always available. It requires only to be diluted and the pH adjusted. Dilution is necessary to avoid inhibition of the yeast by osmotic pressure, and uneconomical utilization of the sugar. A concentration of about 12 percent sugar is satisfactory. The pH is adjusted, usually with sulfuric acid, to around 4.5. This is favorable to the yeast and unfavorable to bacteria. Sometimes nutrients

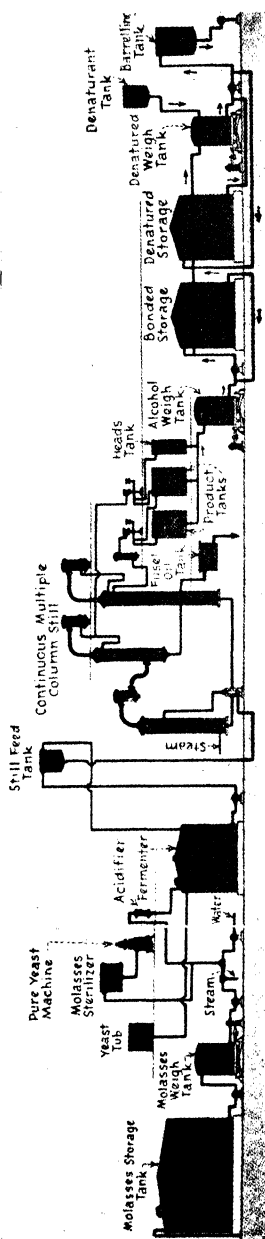


Fig. 285.—Arrangement of equipment in a modern plant for the production of ethyl alcohol by the fermentation of black strap molasses. (From R. R. Collins, *The Lummus Co., Chemical & Metallurgical Engineering*, Vol. 41.)

may be added, especially a source of nitrogen such as ammonium sulfate.

Properly prepared carbohydrate “mashes” are sometimes freed from excess undesirable live bacteria by heat, but this is expensive. The mash is inoculated with an aciduric and alcohol-resistant strain of yeast, the variety depending on the conditions under which the fermentation is to proceed and the exact end-products desired. The inoculum comes from a large tank of carefully maintained pure culture previously inoculated from a smaller seed tank and the latter from a flask or tube of culture (Figs. 285 and 286). The inoculum tank or “yeast machine” is aerated as this promotes rapid growth of yeast cells. The maintenance of purity of the inoculum is a responsibility of the bacteriologist, and woe betide him if some spore-former or “wild” yeast gets in and ruins 100,000 gallons of “mash”!

Fermentation is allowed to continue in vats holding as much as 50,000 gallons for about forty-eight hours at a carefully controlled temperature of about  $25^{\circ}\text{C}$ . until the yeast stops growing due to the concentration of alcohol and other products. (Figs. 287 and 288.) Aeration may be used at first to promote rapid growth, but anaerobiosis is soon established to promote alcohol accumulation and prevent its oxidation to carbon dioxide and water. The temperature tends to rise but is kept down by streams of cool water sprayed on

the fermentation tanks or by other means. Some yeasts can become habituated to growth until the alcohol concentration of the mash has reached as high as 16 percent.

After fermentation the crude alcohol or "high wine," as it is called, is usually a mixture of ethyl alcohol and a small amount of glycerol with fusel oil. The last contains amyl, isoamyl, propyl, butyl and other alcohols with acetic, butyric and other acids, as well as various esters. The high wine is driven off from the mash or "beer" by heat and further purified by fractional distillation.

The chemical reactions involved in the fermentation are very complex. The chief constituents of fusel oil are probably derived from amino acids in the molasses or mash. The large amounts of

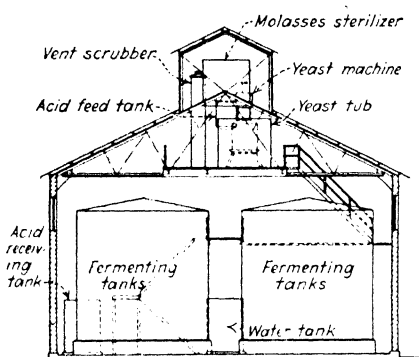


Fig. 286.—Elevation of the fermenting room showing yeast equipment. (From R. R. Collins, *The Lummus Co., Chemical & Metallurgical Engineering*, Vol. 41.)

carbon dioxide evolved are purified and compressed in tanks or made into "dry ice." Part of this may be used for cooling the vats. The common intermediate substances, methyl glyoxal, pyruvic acid and acetaldehyde, take part in the series of reactions, and preliminary combination of the dextrose with phosphoric acid (phosphorylation) occurs.<sup>1</sup>

*Production of Alcohol by Bacteria.*—Certain aerobic bacteria (*Bacillus acetoethylicus* or *Bacillus macerans*) are also capable of producing ethyl alcohol as well as the very valuable substance, acetone, from carbohydrate materials. Molasses, potatoes and other materials are used.

*B. acetoethylicus* is also important in flax retting. It is probably identical with *B. macerans*. It produces the enzyme pectinase.

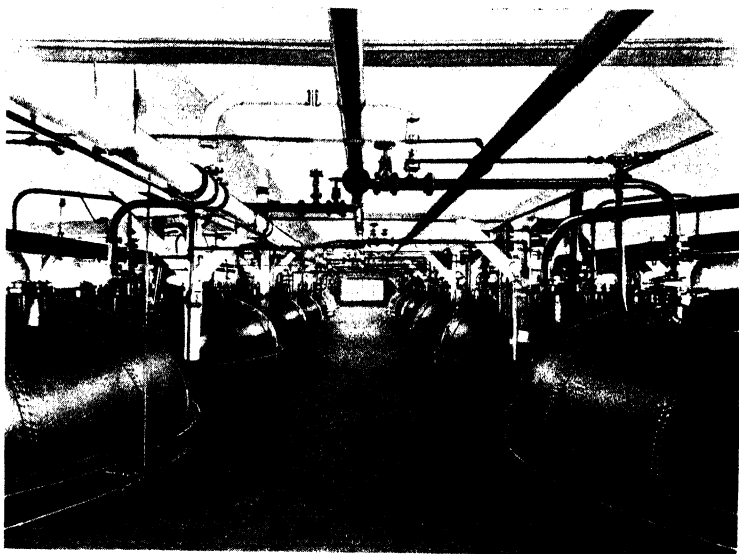


Fig. 287.—Upper level of 50,000-gallon fermentation tanks. (Commercial Solvents Corp.)

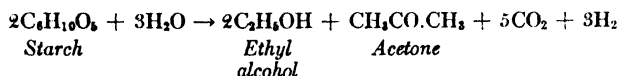


Fig. 288.—Lower level of 50,000 gallon fermentation tanks. (Commercial Solvents Corp.)

The spore-forming aerobes to which it is related are widely distributed in soil, water, feces, etc., and possess properties similar to it. Studies of the biochemical properties of highly fermentative bacteria of this kind may yield more species of even greater commercial value. *B. macerans* is weakly gram-positive, motile and grows well in ordinary laboratory media (see section on genus *Bacillus*, page 500).

For alcohol production by these bacteria cellulose-containing wastes may be hydrolyzed by steam and sulfuric acid. Corn cobs and oat and peanut hulls have been successfully adapted to alcohol production by this means. As in other fermentative processes, conditions of growth and incubation must be made suitable to the organisms involved and productive of the end products desired. For *B. acetoethylicus* nitrogenous materials must be added if not already present, and other foods which the deficiencies of different mashes may require. The process is an aerobic one. The pH for maximum alcohol production by this organism is around 7.0; a pH around 6.0 results in more acetone production. The pH must be maintained by a buffer such as calcium carbonate. A temperature of about 42° C. is suitable. Incubation periods of about six days are required.

The exact chemical processes involved are complex, but in general they resemble some phases of the fermentation of glucose by *E. coli*. The overall reaction appears to be



Certain byproducts like acetic and formic acids which appear in the high wine may be derived from other nutrient substances in the mash, such as amino acids.

*Use of Raw Cellulose for Alcohol Production.*—A method of producing alcohol from raw cellulose by bacterial fermentation has been sought for some time, and with some success as several cellulose-to-alcohol fermenters have been discovered. For example, *Clostridium dissolvens* (*B. cellulosa-dissolvens* of Khouvine)<sup>9</sup> attacks cellulose specifically and will not grow in a medium which does not contain it. Veldhuis, Christensen and Fulmer<sup>10</sup> used a mixture of thermophilic, cellulose-splitting organisms common in horse manure. In developing the fermentative combination of organisms, manure is introduced into a medium of the following composition:



Cellulose (pulped paper) . . . . .	3.0 gm.
NH <sub>4</sub> Cl . . . . .	0.25 gm.
K <sub>2</sub> HPO <sub>4</sub> . . . . .	0.25 gm.
CaCO <sub>3</sub> . . . . .	4.0 gm.
Tap water . . . . .	100.0 cc.

The pH should be about 7.8.

The mixture is held anaerobically at 55° to 60° C. The culture is transferred to new medium every six days. This mixed culture ferments about 99 percent of the cellulose at 60° C. Along with the alcohol there appear carbon dioxide, hydrogen, methane, and acetic, butyric and other volatile acids. This process has great possibilities, as the ratio of the various products may be varied by providing different media, pH, temperature and other factors, and because raw cellulose is a very cheap source of alcohol. It is especially noteworthy that pure cultures of the individual species of bacteria involved in the cellulose fermentation do not act nearly as efficiently as the mixture of bacteria normally present in the manure. Further researches along these lines, in order to develop more of such processes into profitable factory procedures, are desirable.<sup>10a, 10b, 10c</sup>

**Other Processes.**—In another process a mixture of species of cellulose-dissolving bacteria from the gut of a termite (a wood-digesting species of ant-like insect) is selectively cultivated at 45° C. after eliminating thermosensitive forms by heating as described on page 591. The mixture of bacteria is kept active by transferring in a nitrate-cellulose medium containing 20 percent aqueous extract of yeast. The culture will digest corncobs easily. To utilize the culture, the cobs are autoclaved in water at 15 pounds for 45 minutes. They are then dried and added to the nitrate medium in place of cellulose and the mixture is inoculated with the bacteria from the termite. Fermentation continues for about a week and the products are acetic, butyric and lactic acids. In contrast with the manure culture described above, no ethyl alcohol is produced at all.

**Bacteria and Synthetic Rubber.**—The value of specialized research in this field is shown by the development by Lieberman et al.,<sup>11</sup> in 1943, of a variety of organism producing commercially feasible amounts of 2,3-butylene glycol or *butanediol* (see p. 351) used in the manufacture of synthetic rubber. This glycol is formed directly during the fermentation of cornstarch mash. The butanediol is esterified and then fractionally distilled. Combined with styrene from petroleum it forms the basis of the synthetic

gum. The process and type of organism have not been widely publicized.

**Distilled Beverage Industries.**—The manufacture of alcoholic beverages is a time-honored occupation, reference to wine being found in Genesis, when Noah used it and became “drunken” (Gen. 9:21). In Chapter 1 some details are given concerning the part which Pasteur played as the first great industrial bacteriologist in developing methods for the fermentation industries. Today the production of alcohol for manufacturing and beverage purposes by fermentation and distillation is a very valuable and well developed industry. In principle the production of alcoholic distilled beverages is similar to the production of industrial ethyl alcohol. Refinements are introduced with respect to flavor, aroma, color, sanitation, etc., which are not necessary in the making of industrial alcohol.

Carefully selected grain is stored and ground as needed. Rye whiskey is prepared from mash containing at least 51 percent rye, bourbon, at least 51 percent corn. A “spirits mash” consists of 88.5 percent corn, 1.75 percent rye, and 9.75 percent barley malt. The grain, mixed with water, is autoclaved, cooled, diluted, and 1 percent barley malt is added. The malt, containing amylolytic enzymes, hydrolyzes the starch. The “mashing,” or hydrolysis, proceeds in a special tank, at around 65° C., for about thirty minutes. The mash is then pumped to the fermentation tanks. Here it is heavily inoculated with selected yeast which has been cultivated in a mash previously made somewhat acid (*pH* 4.0) with lactobacilli as follows.

The yeast culture medium consists of mash similar to the main mash, but more concentrated. It is hydrolyzed with maltase, inoculated with lactobacilli, and allowed to ferment till the *pH* reaches about 3.9. It is then heated to around 82° C. to stop the fermentation and kill the lactobacilli, cooled to 24° C. and inoculated with the yeast. The original sugar content of around 24 percent is finally reduced to about 12 percent by the yeast and bacilli.

As the main mash enters the fermenter, the yeast mash mixes with it. Water is added so that the final mash concentration is 36 gallons of mash per bushel of grain. The *pH* is adjusted by means of sulfuric acid, or acid residue from previous distillation, to about 4.9 at which point the remaining malt in the mash acts on the dextrins, etc. Contamination is also discouraged by this degree of acidity. Fermentation is complete in about seventy-two hours, as in industrial alcohol production.

In the distillation of alcoholic beverages, the fermented mash ("beer") is first distilled *in vacuo*. Aldehyde, esters and fusel oil are then separated from the "high wine" by further vacuum distillation. The purified product is then concentrated to about 95 percent alcohol and further purified by fractional distillation *in vacuo*. The remaining details of distillation, blending, etc., do not involve microbiological principles.

The bacteriologist maintains the yeast and *Lactobacillus* cultures; arranges precautions against contamination of the yeast culture vats, fermenters, etc.; makes frequent cultural checks on the various mashes; and examines the bacteria in the air, grain, water supplies, etc. Special investigations and "trouble shooting" add to his work.

**Production of Butanol and Butyric Acid.**—As mentioned in the section on the genus *Clostridium*, there are numerous species of soil-inhabiting, spore-forming anaerobes which ferment carbohydrate substances with the production of butyric acid, butyl alcohol and other substances of enormous value in drugs, paints, synthetic rubber, explosives, plastics, etc. Some species produce isopropyl alcohol and acetone as well. Important among these organisms are *Cl. acetobutylicum* and *Cl. butylicum*. The true identity of the latter organism seems questionable.

As in the production of acetone and ethyl alcohol, carbohydrate mashes may be prepared from sugars, starches or cellulosic materials by appropriate processes. Corn is commonly used, after being degermed, ground to meal and autoclaved with water at 30 pounds pressure for at least two hours. This alters the starch and sterilizes the mash. After diluting to about 10 percent corn meal and cooling to around 40° C., the pH is adjusted to about 6.0, and a pure culture of the selected organism is introduced with aseptic precautions.

Complete sterilization of all apparatus is essential because many undesirable bacteria of the environment can grow well under the conditions of the fermentation. Particularly troublesome are species of *Lactobacillus*. An organism called *B. volutans*, a gram-positive, nonspore-forming rod, is also especially dangerous.

Fermentation proceeds anaerobically for about three days, when the high wine is removed and subjected to fractional distillation to separate the various products of the fermentation. The nature and proportions of these depend on the strain of organism used, the composition of the mash, the pH, and other factors. Normally butyl alcohol, acetone and ethyl alcohol, with carbon





Fig. 289.—Flow sheet of Industrial ethyl alcohol manufacture. (Courtesy of the U. S. Industrial Chemicals, Inc., N. Y. C.)



dioxide and hydrogen in large amounts, predominate when *Cl. acetobutylicum* acts in a glucose mash. Other substances may occur in smaller amounts. Riboflavin, (a vitamin of the B complex) is a valuable constituent of the residue after distillation of the solvents. Butyl and isopropyl alcohol are important among the fermentation products of *Cl. butylicum*, along with butyric and acetic acids.

*Relation of Sporulation.*—The most vigorous and productive fermentations are brought about by cultures of the organisms which have undergone a series of heat treatments to select vigorous and rapidly spore-forming cells. A long process of alternate heatings to kill vegetative cells, and periods of vegetation and sporulation which are checked abruptly by heating again, select the rapid and resistant sporulating survivors. Temperatures of around 100° C. for about one minute are used. Thus only vegetative cells and less resistant spores are killed and the rapid producers of very resistant spores are selected for use.

**Production of Lactic Acid**—Lactic acid is used in a large number of commercial processes, such as the preparation of drugs, acidification of wines for vinegar production, pickles, preparation of foods, plastics, lacquers, etc. It is produced by many fermentative organisms from various carbohydrate substrates such as potato or corn starch, molasses or milk whey. The most commonly used organisms are *Streptococcus lactis* and lactobacilli (*L. delbrückii*, *L. leichmannii*, *L. bulgaricus*). As in other industrial fermentations, any nutrient deficiencies in the mash must be rectified by appropriate additions of sources of phosphorus, nitrogen, or whatever may be needed. For lactobacilli, the vitamins, pantothenic acid and riboflavin as well as nicotinic acid are requisite additions unless the mash contains these in adequate amount (see section on assay of vitamins with lactobacilli, pages 598, 599).

In the United States corn, molasses and whey are commercially practical sources of carbohydrates, as in other important industrial fermentations. Indeed, the general principles illustrated by the processes for producing ethyl and butyl alcohols hold true in lactic acid production, modifications being made in the composition of the mash, time, temperature and pH of the process in order to favor the organisms used and to promote lactic acid formation. For the lactobacilli temperatures around 45° C. are used, for *Str. lactis*, about 25° to 30° C. Anaerobic conditions are suitable for both genera. A pH of around 5.5 favors growth of lactobacilli; around 7.0 is suitable for *Str. lactis*. Fermentation times vary with

substrates, organisms and other factors. Metal vats are not used because of the acidity of the "beer."

Lactic acid production differs from previously described fermentative processes in that the acid is neutralized with calcium hydroxide as fast as it is formed. Otherwise growth would soon cease. The final product in the fermentation vats is therefore calcium lactate which, if desired as pure acid, must be changed to lactic acid by the addition of sulfuric acid which precipitates the calcium as calcium sulfate. Calcium lactate as such has many commercial and medicinal uses.

As in the production of butyl alcohol with species of *Clostridium*, precautions are necessary to prevent contamination of the mash used for lactic acid production. As the anaerobic conditions and substrates favor growth of clostridia, special precautions are taken to avoid these organisms.

These examples serve to illustrate the general nature of pure-culture commercial processes. Regardless of end-products, the same general principles apply to all.<sup>1</sup> The bacteriological applications lie in discovering organisms which produce substances of value, the optimum conditions for their growth, and in maintaining pure cultures of maximum productive power. It is obvious that, as Pasteur discovered in the production of wines and beer, there are "diseases" of the various industrial fermentative processes which are due to overgrowth by contaminating organisms. These may suppress the growth of the desired bacteria or produce other acids or alcohols than those desired, or metabolize those sought as fast as they are formed, thus reducing the yield. Special preliminary treatments such as heating may be employed, or special pure-culture technics in some processes.

Turning now to fermentative processes in which pure cultures are not necessary, or in which they are, in practice, seldom used, we encounter a number of procedures which involve bacteria but usually no special knowledge of bacteriology. Very often, however, the skilled bacteriologist is in great demand when trouble occurs in such processes. Vinegar manufacture is one of these processes and has been described.

**Ensilage.**—One of the commonest of these fermented products is ensilage. Fermentation of the carbohydrates in green plant tissues, especially by members of the genera *Lactobacillus*, *Escherichia*, and *Aerobacter* is used constantly (and usually unconsciously) by farmers in the preparation of silage, a food for cattle. Finely chopped, partly mature plants like corn stalks or



alfalfa are tightly packed in tall, cylindrical tanks (silos). As fermentation proceeds, the material becomes warm and acid. The heat can be reduced if the rate of oxidation is decreased (exclusion of free oxygen) by tight packing. Oxygen is used up rapidly so that molds and strict aerobes cannot grow, only facultative and strict anaerobes surviving. The acidity prevents the growth of putrefactive organisms. In the first stages probably the *Escherichia* and *Aerobacter* predominate; however, this is undesirable as they produce gas. As acidity increases, they subside and the aciduric organisms (chiefly lactobacilli and streptococci) predominate. These produce lactic acid, with small amounts of butyric, propionic and acetic acids, esters, etc., which give an aroma and flavor to ensilage which is relished by cattle.

After three or four weeks, the process slows and the fermented mass gradually cools. Carbon dioxide is produced during the process and often settles in the lower part of silos so that a person ignorant of this may die if he stays in the depths of a poorly ventilated silo.

It has been claimed that fermentation in silos may be facilitated by introducing cultures of various fermentative bacteria such as *Streptococcus lactis* or *Lactobacillus sp.*, as the material is packed. However, this is seldom done in practice. Various other organisms are doubtless involved, including the bacteria (micrococci, spore formers, *Pseudomonas*, etc.) of the soil. In some sections of the country molasses is added to promote fermentation by the acid formers, and improve palatability but this is not a common practice.

If too much soil is introduced with the fodder undesirable and excessive putrefactive processes spoil the product. For example, butyric acid organisms like *Clostridium butyricum* get in and ruin the silage. The action of such organisms would constitute a "disease" of silage. *Cl. botulinum*, a soil anaerobe, forming a deadly poison, has also caused much damage to livestock by its growth in silos.

**Sauerkraut.**—The production of this savory delicacy is dependent upon the acid fermentation of cabbage by the bacteria normally present on the plants. These include the lactobacilli, soil streptococci and other soil bacteria. The desired fermentation is principally lactic.<sup>12</sup> Pure cultures of lactobacilli are sometimes used to aid the process.<sup>13</sup> Commonly, however, the fermentation is allowed to proceed naturally. Salt is placed between the layers of shredded cabbage as it is packed and this prevents the growth of many

undesired bacteria and also draws out the juices of the cabbage. Except for the salt, sauerkraut is quite analogous to silage. Sometimes "sauerkraut" is prepared by merely cooking cabbage in vinegar. Such a product lacks the true, rich flavor of fermented sauerkraut because the natural fermentation produces many organic esters, etc., besides acetic and lactic acids, which add greatly to the aroma and flavor.

*Making Sauerkraut at Home.*—Directions for domestic sauerkraut will illustrate methods of applying principles used in commercial practice.<sup>14</sup> Large, solid mature heads of cabbage are allowed to wilt for one or two days, so that long shreds may be cut. Any soil or foreign matter must be removed. The shredded cabbage is packed in clean 5-gallon jars or barrels which have previously been paraffined on the inside. Contact with wood may darken the kraut. Salt is sprinkled lightly in, as the packing proceeds, in the proportion of about 2 to 2.5 percent of the total weight. Too much salt may darken the kraut or interfere with fermentation. Too little will permit spoilage by soil bacteria. The kraut is firmly pressed down with a wooden tamper, care being taken not to bruise it, the object being merely to force out the air. Air pockets permit spoilage bacteria to grow and darken the product. Bacteria begin to ferment the carbohydrates in it. When the container is full, the juice should just cover the cabbage. The kraut is covered with a clean muslin cloth and then a paraffined cover just small enough to fit inside the container is put on. A weight may be placed on the cover so as to keep the kraut just below the juice, especially when gas bubbles begin to form. After two to four weeks at summer temperatures, fermentation is complete and the kraut is ready to ship or consume.

If not used at once, it is best heated to about 125° F., put up in jars or cans and finally heated in boiling water for about twenty minutes, after which the containers are sealed and placed in a cold room.

*Bacteriology of Sauerkraut.*—Since the microbial flora normally present on the cabbage leaves is allowed to grow as it can, the bacteriology of sauerkraut fermentation is complex. The conditions of packing provide a rather selective medium so that only certain species are very active. Those sensitive to more than 2 to 2.5 percent salt content are inhibited, as well as the strict aerobes. As in silage a few members of the *Escherichia* and *Aerobacter* groups may start to grow. These are highly undesirable, however, and every effort should be made to avoid admitting them to the

kraut in excessive numbers. Ordinarily, unless spoiled or dirty cabbage is used, acid formation begins almost immediately and increases rapidly, at temperatures around 70° F., and only facultative, anaerobic, aciduric and acidophilic forms can survive. During the first two to five days, certain species of streptococcus-like organisms, somewhat like *Str. lactis*, are found in great numbers. These belong to the genus *Leuconostoc*, *L. mesenteroides* being common. Later, these die off because of the acidity and organisms of the genus *Lactobacillus* because of their greater resistance to acid, gain the ascendancy, such species as *L. plantarum* and *L. brevis* (pento-aceticus) being commonest, in the order named. The latter is most aciduric and predominates finally. Acetic and lactic acids, carbon dioxide and alcohol are produced in considerable amounts. Other acids and esters give pleasant aromas and flavors. Cool summer temperatures around 70° F. favor the best fermentations. Higher temperatures may induce darkening due to abnormal fermentation.

**The Vegetable Fiber Industry. Flax, Hemp, etc.**—The leaves or bark of flax and hemp plants are prepared for use by allowing bacteria which attack *pectin* to act upon them. Pectin is a plant gum which acts as a sort of glue or cement, holding plant cells, tissues and fibers together. When it is hydrolyzed by bacteria, as in the preparation of jute, hemp or flax for manufacture, the bast fibers of the plants are loosened and freed from the non-fibrous parts and are then ready for further treatment. The process is called “retting” and is of two kinds, “dew retting” and “steeping” or “water retting.”

In *dew retting* the fibrous plant material is spread about in piles in meadows. The dew keeps it moist and the natural bacterial action proceeds. The piles of plant stalks or leaves are usually left exposed to the weather all winter, or from 2 to 5 days in warm weather. Unduly warm weather may cause excessive bacterial action, especially by cellulose-digesting molds and bacteria, like *Aspergillus*, *Cellvibrio*, *Cellfalcicula* and various species of *Clostridium*, which may weaken or entirely ruin the fiber.

*Steeping or water retting* may be carried out in slow flowing, warm streams (Fig. 290). The shallows of the Nile have served for retting flax for centuries. The rate and effectiveness of the process depend on the nutrient substances in the water, its temperature and the bacterial flora. As in dew retting, there is little control over the process.

*Tanks or vats* are preferable and are used on a large scale in some

areas. An aerobic or anaerobic flora may be made use of since pectin-digesting species of either group exist. In the anaerobic vat process the vegetable material is placed in the vat which is then filled with water. Soluble nitrogenous and carbohydrate extractives from the plants pass into the water, along with mineral and other nutrient compounds, and constitute a suitable solution for the growth of bacteria. The normal flora of the plants grow in the solution, first the aerobes, which use up the oxygen, then the anaerobes. These include such organisms as *Clostridium butyricum*



Fig. 290.—Flax retting in a stream. (W. D. Darby, "Linen, The Emblem of Elegance," Dry Goods Economist, Copyright by Textile Publishing Co.)

and related soil species, important among which, in this connection, is *Cl. felsineum* which is particularly active in pectin digestion. The process goes on well at warm summer temperatures (around 30° C.). The time is important, as over-retting may permit excessive action of cellulose digesters which ruin the fibers. About two to three days at 30° C. produces satisfactory results.

In the aerobic process, air is bubbled through the tank and aerobic pectin digestion takes place. *Bacillus macerans* is important in this process. It is said to yield a superior grade of flax fiber.

**Bacterial Enzymes.**—Both amylolytic and proteolytic enzymes are produced in sufficient quantity by certain bacteria to be of commercial value. A good example of commercial enzyme production is seen in the production of amylase and protease by *Bacillus mesentericus*. The amylolytic enzyme is used in the brewing and related industries to convert raw starches into materials fermentable by yeasts. Amylases are also prepared from *B. macerans* and *B. polymyxa*.<sup>15</sup> In the textiles industry starch partly digested by bacterial amylase is used for sizing cotton fibers, while similar enzymes are used for desizing cotton prior to dyeing and bleaching processes. Sizing pastes for paper are prepared from starch treated with bacterial enzymes. Bacterial protease, present in preparations of *B. mesentericus*, which is both proteolytic and amylolytic (see section on genus *Bacillus*, page 509), also is used in desizing rayon which is treated with gelatin or casein, as well as for degumming raw silk. There are many other uses in industry for bacterial enzymes.

**Production.**—Amylolytic and proteolytic enzymes from *B. mesentericus* are prepared in culture medium made from soybean and peanut cake, dextrins, and suitable mineral supplement. The medium is sterilized by autoclaving and inoculated with a selected strain of the organism to be used. Some varieties produce more protease and less amylase, and vice versa. Culture media and conditions also have a determining effect on the relative proportions of the two types of enzyme.

The inoculated medium is run into numbers of shallow trays stacked in a cabinet. This favors aerobic growth in a thin surface film. The amount of aeration and temperature are carefully controlled. From 500 to 1000 gallons of "mash" may be incubated at one time. After about a week at around 30° C. the mash is centrifuged at high speed, and the clarified fluid collected and stored at low temperature or preserved with disinfectants. It may be concentrated by distillation in vacuo. The product is of value in commercial processes operating at a pH range between 6.5 and 8, and at all temperatures from around 20° to almost 100° C. In the presence of starch, at pH of 7 to 8, it even withstands boiling for a few minutes.

**Pectinase.**—Pectinase, the enzyme responsible for retting or the hydrolysis of pectin, is formed by a number of bacteria, some of which are used in pure culture under carefully controlled tank procedures and added to the tanks in the form of "starters." Among these are aerobic organisms like *Bacillus subtilis* and a

related species called *B. comesii*. *Clostridium felsineum*, *Cl. butyricum* and its congeners are used for anaerobic retting. Some of the plant pathogens, as *Erwinia carotovora*, are also active producers of pectinase, but are not ordinarily used in retting. If the enzyme could be prepared cheaply in a pure and concentrated form, it might be of great assistance in retting. The same is true of other industrial processes involving bacterial enzymes. Methods of producing pectinase have been devised but are expensive.

**Assay of Vitamin B<sub>1</sub> by Yeast Fermentation.**—(See sections on bacterial metabolism, and on lactobacilli, pages 359, 365.) The rate of fermentation of glucose to carbon dioxide and alcohol by the yeast *Sac. cerevisiae* is largely influenced by the amount of thiamine (B<sub>1</sub>) available. In a synthetic medium this vitamin may be supplied or withheld at will and the effect of exact amounts of thiamine determined by measuring the CO<sub>2</sub>. By the addition to the synthetic medium of graded amounts of various "unknowns" such as foods, these may be tested for thiamine content. Certain related compounds also found in various foods have an action similar to thiamine. These must be measured separately in a sample of the unknown treated with sodium sulfite which destroys the thiamine but not the interfering substances. The difference between results obtained in fermentation tests with the treated sample and the untreated sample represents true thiamine. The CO<sub>2</sub> volumes are then compared with parallel experiments in which known amounts of pure thiamine were used, and the thiamine content of the food is easily computed.

In preparing materials for the assay, as described by Schultz and his co-workers,<sup>16</sup> one prepares a buffer solution:

(A) Water	1000.0 cc.
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	180.0 gm.
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	72.0 gm.
Nicotinic acid	0.2 gm.
Sterilize at 100° C. for 30 minutes.	

and the medium (B)

Glucose	200.0 gm.
MgSO <sub>4</sub>	0.1 gm.
KH <sub>2</sub> PO <sub>4</sub>	2.2 gm.
KCl	1.7 gm.
FeCl <sub>3</sub>	0.01 gm.
CaCl <sub>2</sub>	0.5 gm.
MnSO <sub>4</sub>	0.01 gm.

The medium is mixed and then sterilized intermittently. The yeast inoculum consists of 10 gm. of bakers' yeast (not the edible cakes) freshly suspended evenly in 200 cc. of water.

Of the medium (B) 7.55 cc., and of buffer (A) 2.5 cc., are placed in flasks held in a water bath at 30° C. Arrangements are made for shaking and for collection of the evolved carbon dioxide. Machines are available for this purpose (see Fig. 291). The sulfite-treated unknown is placed in one flask, the untreated unknown in another. The volume of each is made up to 40 cc. with distilled water and 10 cc. of the yeast suspension added and the flasks

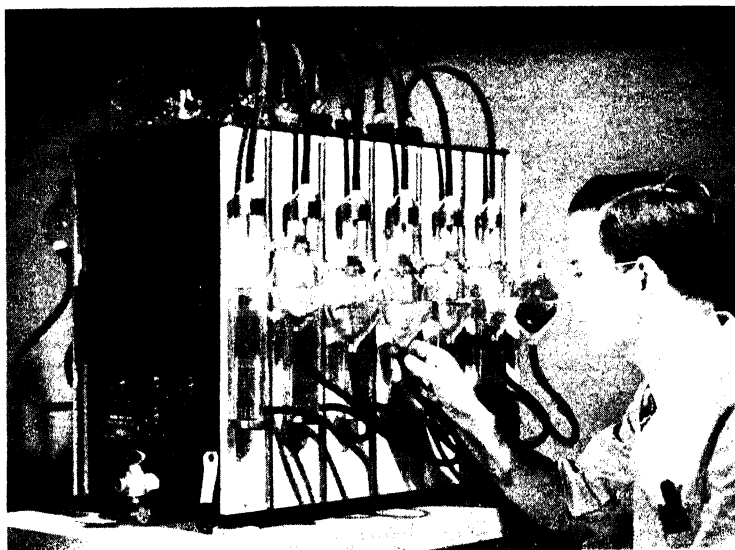


Fig. 291.—Yeast machine for thiamine or vitamin assay by method of Schultz. (The Fleischmann Laboratories, Standard Brands Inc.)

closed *quickly*. Incubation and shaking continue for three hours, and gas-volume readings are made in gas burettes attached to the fermentation flasks. From readings of the two unknowns, compared with readings of similar flasks containing known amounts of thiamine, the amount of thiamine in the food is computed.

**Use of Lactobacilli in Vitamin Assays.**—Lactobacilli (see chapter on bacteria in milk, page 565) are heterotrophic bacteria and absolutely require complex organic substances for growth, as for instance, pantothenic acid and riboflavin. As shown by Strong,

Snell, Peterson and their colleagues,<sup>17</sup> the amount of acid formed from glucose by the lactobacilli depends on the presence of these vitamins as well as other substances. By providing a medium complete in all respects but deficient in, say pantothenic acid, one may measure the amount of this vitamin in various foodstuffs by adding the foodstuff under investigation to the culture medium in measured amounts and then measuring the amount of acid formed from glucose in the medium after incubation. A basal medium contains hydroxide-treated peptone, sodium acetate, cystine, asparagine, riboflavin, 1 percent glucose, and yeast supplement, as well as suitable minerals including phosphates, iron chloride, magnesium sulfate, etc. It is deficient in pantothenic acid.

The assay cultures are prepared in tubes, the foodstuff to be tested added, and the total volume made up to 10 cc. Tubes containing graded amounts of pantothenic acid solution of known strength in place of the food serve for standards of comparison. All are sterilized, and a drop of heavy culture of *Lactobacillus helveticus* is added to each. After about seventy-two hours of incubation, the acid in all tubes is titrated, plotted on charts, and the pantothenic acid content of the "unknown" determined by comparison with the standards. The method is accurate and saves much time and money as compared with the use of rats and chicks.

**Other Processes. Use of "By-products."**—A considerable number of other processes of industrial importance depend on the action of bacteria, but a detailed treatment of them here would be impossible. Some of them are not thoroughly understood. Oxalic acid, glycerin, amyl alcohol, humus, acetone, many substances used in paints, drugs, photography, and a long list of other substances and processes are produced commercially by microorganisms. Waste products such as sawdust, shavings, garbage, beet-pulp and, in fact, almost any fermentable or putrescible substance, can be made to yield valuable products by treating them with the proper bacteria, yeasts or molds. The possibilities are limited only by the extent of man's knowledge of microorganisms and his ingenuity in making use of them. Even combustible gases consisting of methane and hydrogen can be made, but the bacterial processes cannot at present compete with natural or coal gas. The use of waste carbon dioxide from alcohol manufacture is a valuable industry in itself.

**Industrial Spoilage.**—In contrast with the useful activities of bacteria, a word may be said of their destructive action. Several



causes of industrial spoilage have been mentioned in this chapter and in the chapters on soil and water bacteria. *Micrococcus*, *Alkaligenes*, *Flavobacterium*, *Serratia*, yeasts, molds, etc. are common causes of spoilage, but each type of product is attacked by certain species of microorganism which can metabolize the substance especially well. For example, spoilage of cellulosic products, such as wood, paper, fibers, tobacco, cotton, etc., is brought about by cellulose decomposers like *Cellulomonas*, *Cytophaga* and anaerobic organisms of the soil. Fermentable substances like syrups, beverages, etc., are attacked by yeasts, lactobacilli, organisms of the coli-aerogenes group and various environmental bacteria including the genus *Clostridium*. Spoilage of proteins like meats, fish, milk and so on results from the action of putrefactive species such as *Pseudomonas*, *Bacillus*, *Proteus*, *Micrococcus*, *Clostridium* and many others. ZoBell and Grant have shown that rubber may be attacked by certain bacteria.<sup>18</sup>

Prevention of spoilage depends on maintaining conditions unfavorable to organisms which can grow on or in the particular product involved. This may involve drying, refrigeration, aeration, the use of inhibitory salt, sugar or acid concentrations, radiation with ultraviolet light, exposure to sunlight, treatment with substances like creosote, sodium benzoate and the like. Dimond and Horsfall have suggested a method of preventing bacterial spoilage of rubber.<sup>19</sup> Each type of spoilage is a problem in itself and research in such fields, as well as in the field of productive microbiology, will prove interesting and may prove lucrative to those sufficiently interested to make a special study of the matter. A thorough knowledge of selective bacteriostatic methods is valuable in this field.

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## DIPLOCOCCUS PNEUMONIAE

**Genus *Diplococcus*.**—British writers refer to the pneumococci as *Streptococcus pneumoniae*. Not only do the pneumococci often form chains (usually made up of from two to eight *pairs* of cocci) but, like some of the beta-type hemolytic streptococci, they are encapsulated. In blood-agar plates they produce the alpha type of reaction, often with considerable hemolysis. They are facultative, heterotrophic and parasitic. Methods of cultivation and study are like those used for streptococci. *Diplococcus pneumoniae*, the so-called “pneumococcus,” is the only important species in this genus. It is found in the saliva and sputum of patients with *lobar pneumonia*, and also occurs frequently in the normal mouth and throat.

**Distinguishing Characters of the Pneumococci.**<sup>1</sup>—The cocci are rarely spherical, having the form of short projectiles placed base to base. They are often referred to as lance-shaped but they are conical and not flat. They are gram-positive. Pneumococci as a rule are *extremely pathogenic for white mice*. Advantage is often taken of this fact to isolate pneumococci from sputum of patients for diagnosis. The sputum is injected into the mice intraperitoneally. After six to twenty-four hours the mice die or become very ill, and enormous numbers of pneumococci are found in the peritoneal cavity and heart blood. Most or all of the other bacteria normally in the sputum are taken up by leukocytes or otherwise destroyed (Fig. 292). The heart blood or peritoneal exudate of the mouse may be streaked on infusion-blood-agar plates and *Diplococcus pneumoniae* isolated in pure culture. The colonies are thin, flat, translucent, greenish and from 0.5 to 2 mm. in diameter.

The cocci found on the peritoneum of the mouse may be identified tentatively by their *morphological* peculiarity and by their *capsules* (Fig. 293) containing carbohydrate which gives type specificity to the cell. These capsular carbohydrates react specifically with antisera prepared by injecting animals with them.

Pneumococci are further differentiated from all other similar bacteria by the phenomenon of *bile solubility*. To determine this property, the peritoneum of the mouse is well washed by means of a fine-tipped pipette and small rubber bulb, with 1 or 2 cc. of salt solution, and the washings collected in a small tube. After the mucus and leukocytes have settled out, the supernatant fluid, turbid with pneumococci, is transferred to another tube and

about one-fourth of its volume of ox-bile (or, far better, 2 percent solution of sodium lauryl sulfate) added. In a few minutes the fluid will become clear, due to the fact that the pneumococci are entirely dissolved. Broth cultures will exhibit the same phenomenon and are perhaps more convenient to work with. Some strains of pneumococci are more readily soluble than others. If necessary, further identification can be made by testing the ability of pure cultures of the organisms to ferment inulin (see Table X). Pneumococci are the only streptococcus-like organisms, highly pathogenic for mice, and bile soluble, which *ferment inulin* and produce the alpha type of appearance in blood-agar plates.

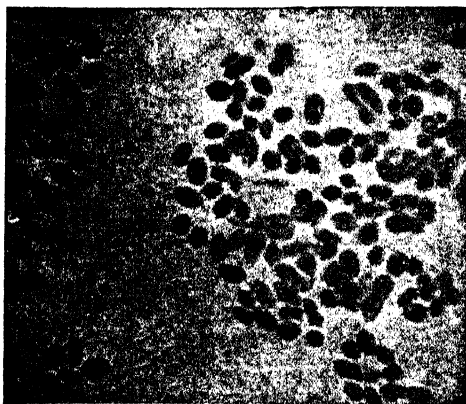


Fig. 292.—Pneumococci with capsules in a smear preparation from peritoneal exudate stained by W. H. Smith's method;  $\times 1500$ . The capsules are swollen by contact with type specific serum. Cf. Fig. 293. (W. H. Smith; photo by L. S. Brown.)

**Serological Types of Pneumococci.**—A long study of pneumococci has shown that they may be divided into thirty-two or more serological types which are designated with Roman numerals.<sup>2</sup> These are analogous to the Lancefield groups of streptococci and, as in the streptococci, serological specificity is conferred by a carbohydrate. Pneumococci do not possess a type-specific M substance so far as is known. Immune sera are available representing each type.

If the peritoneal exudate from a mouse, obtained as described above, or the fresh sputum from a patient with pneumonia, is mixed with each of these thirty-two sera, in separate tubes, or in droplets on microscope slides, *one* tube or slide preparation very often shows, after a few minutes, characteristic reactions including

*agglutination* of the cocci, *precipitation* of specific soluble substance derived from the capsules, and *swelling of capsules* (Figs. 292, 293). This swelling of the capsules is spoken of as a "*quellung*" (German for *swelling*) reaction. It was first described by Neufeld and is sometimes called the Neufeld reaction. Thus, we may say that the patient is infected with type I, II, III or some other type of pneumococci, according to type of serum which causes these reactions.

The procedure of determining the serological type to which pneumococci belong is often spoken of in the laboratory as "typing." It requires considerable experience.<sup>3</sup> There are numerous methods of determining pneumococcus types.<sup>4</sup>

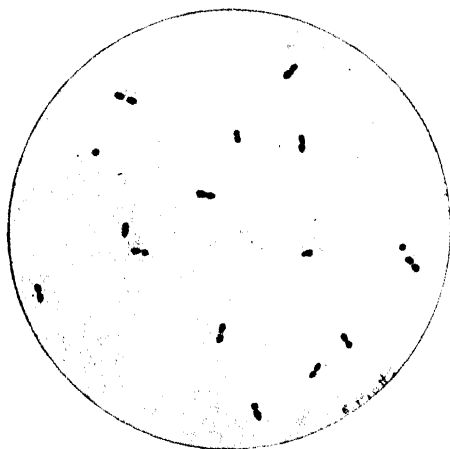


Fig. 293.—Appearance of negative quellung reaction showing capsules not swollen. (From Lord and Heffron, "Pneumonia and Serum Therapy." Courtesy of The Commonwealth Fund.)

The "typing" of pneumococci is of great therapeutic importance because an efficient antiserum exists for type I infections, and for some other types as well, especially II, IV, V, VII and VIII.<sup>5, 6</sup>

**Transformation of Pneumococcal Types.**—A very interesting agent capable of inducing a permanent alteration in a bacterial cell is a desoxyribonucleic acid fraction extracted chemically from type III pneumococci.<sup>10</sup> When rough variants of type II strains are cultivated in contact with minute amounts of the desoxyribonucleate, they are permanently changed into a smooth variant having voluminous, type-III-specific capsules. It is significant that the inducing substance and the substance produced in response to its presence are chemically distinct. *Only actively*

growing type II cells undergo the change to type III; resting cells are not affected. It is suggested that the inducing agent "interacts with the R cell giving rise to a coordinated series of enzymatic reactions that culminate in the synthesis of the type III capsular antigen." It is interesting that the newly transformed cells contain large new quantities of the type-specific transforming substance, so that this substance is *actually multiplied as part of the specific heredity mechanism* of the altered cells. These facts, and others, "indicate the implications of the phenomenon of transformation in relation to similar problems in the fields of genetics, virology and cancer research."

**Soluble Specific Substance of Pneumococci.**—As shown by Avery, Heidelberger and others, our ability to classify pneumococci as types I, II or any other, depends on the presence, in their capsules, of a peculiar carbohydrate-protein complex which can be extracted by appropriate solvents and which is characteristic for each type.<sup>7</sup> This carbohydrate in a pure state is spoken of as a soluble specific substance ("S. S. S.") and it is this substance, coupled with the cell proteins, which produces the antibodies in the type-specific sera used in diagnosis, and lends *specificity* to the reactions by which the organisms are divided into types. If deprived of their capsules, as when they vary toward the rough phase, they are immunologically indistinguishable. They are also avirulent. Compare this with streptococci and typhoid bacilli (pages 544 and 483).

These facts relating to the occurrence of specific carbohydrate substances in or on cells of bacteria are of much wider than medical interest because they illustrate a phenomenon of fundamental importance which is encountered again and again in bacteriology—the occurrence among members of a species, of immunological "types" or groups which differ because of the chemical differences in their carbohydrates. The beta-type hemolytic streptococci described in a previous chapter furnish one example, the influenza bacilli another (see pages 546 and 634).

**Relations of Capsules to Virulence.**—Virulence of pneumococci is definitely associated with their capsules. In general, encapsulated organisms (if they have invasive powers) seem to be less readily taken up by leukocytes and also to be protected from the action of antibodies. The capsular substance also appears to be toxic *per se* as in type B influenza bacilli and pneumococci. Type III pneumococci have particularly heavy, mucoid envelopes or capsules and were formerly known as *Streptococcus mucosus* or *Pneumococcus*

*mucosus*. Probably because of their heavy capsules, type III pneumococci produce a particularly dangerous form of pneumonia, once they gain a foothold. A striking parallelism in this respect is seen between pneumococci and influenza bacilli of type B, as well as anthrax bacilli and *Clostridium perfringens*.

**Pathogenic Action of Pneumococci.**—Pneumococci are highly invasive organisms and, like beta hemolytic streptococci, can infect many different parts of the body and, according to their localization cause meningitis, septicemia, peritonitis, empyema, sinusitis, etc. The name pneumococcus is, of course, derived from the fact that they are frequently seen as the cause of pneumonia.

*Pneumonia.*—The term “pneumonia” includes any infectious or pathological processes in the lung, but we shall confine our discussion of it to the disease *lobar pneumonia* caused by pneumococci. These organisms are commonly present in the throat and nose of normal persons, especially serological types III, VI and XXVIII, although this varies greatly in different places and seasons. The types most commonly involved in actual disease are I, II, IV, V, VII and VIII. In very young children types XIV and XIX are also frequent, with variations in respect to district and season.<sup>1, 6, 8, 9</sup>

When these cocci gain a foothold in the lung, they grow in the alveoli and cause an irritation and a pouring forth of plasma and bloodlike fluid into the air spaces of the lung. This fluid eventually fills the air spaces, and deprives the patient of the use of the affected portion of the lung. Usually one or more whole *lobes* (hence *lobar*) are affected at a time, and the patient may then die. If his resistance is good, he may survive, especially if given type-specific antipneumococcus serum, or one of the drugs of the sulfonamid group. He does not develop any durable immunity.

The method of transmission of pneumonia is chiefly through droplets of infected saliva and nasal and pulmonary mucus, and by inhalation of infected dust. Kissing undoubtedly transmits the infection but obviously not every such infection results in disease. Romance has a powerful ally in natural resistance to infectious disease. Pneumococci survive desiccation very well, and thus are found in dust in patients' rooms, hospital wards, etc.

The organisms are present in almost everyone's nose and mouth, but in order to produce disease appear to be obliged to await a reduction of the resistance of their victim by some other means, especially previous disease such as influenza, whooping cough or measles, or exposure to cold, starvation or great fatigue. Much

fatal pneumonia occurs in very young children following diseases of childhood. Avoidance of exposure to such diseases in infancy (or under six years of age) is imperative. For adults, *especially elderly persons*, avoidance of contact with pneumonia patients and of excessive exposure and fatigue are important.

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## CHAPTER 38

### GENUS NEISSERIA

THE COCCI of this genus comprise a rather closely integrated group of organisms which exhibit some startling contrasts and similarities among themselves. Information brought to light by Carpenter and his colleagues has somewhat altered older conceptions of the relationship between some of these organisms and diseases which they cause.

The family Neisseriaceae includes two genera, *Neisseria* and *Veillonella*. The latter are small, gram-negative, anaerobic cocci



and occur chiefly in the normal mouth. Only two species are described. The group has been relatively little studied and will not be discussed in detail.

**Genus *Neisseria*.**—This genus contains eleven species as at present classified. The genus derives its name from a German bacteriologist, Neisser, who, in 1879, discovered and studied one of the two most important species, *N. gonorrhoeae* (the cause of gonorrhea).

The *Neisseria* are rather small, gram-negative diplococci, each cell characteristically flattened where it is in contact with its mate, each cell having somewhat the shape of a coffee bean (Fig.

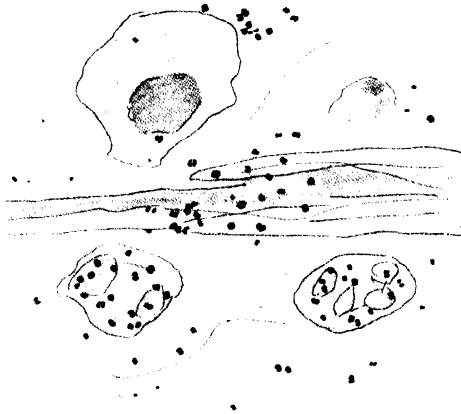


Fig. 294.—Typical organisms of the genus *Neisseria* in pus ( $\times 900$ ).

294). They are aerobic and grow best at  $37^{\circ}$  C. All but one, *N. gonorrhoeae*, are inhabitants of the normal respiratory tract.

Of the eleven listed species of *Neisseria*, three commonly cause disease. *N. gonorrhoeae* causes gonorrhea, while *N. intracellularis* and, as shown by Branham, *N. flavescens* cause epidemic meningitis; however, *N. flavescens* is encountered only occasionally as the cause of this disease. *N. catarrhalis*, as its name implies, was originally thought to be involved in catarrhal conditions of the upper respiratory tract, but this is now open to considerable doubt.

The gonococcus and meningococcus are highly selective and fastidious in the matter of artificial culture conditions,<sup>1, 2, 3, 4</sup> requiring blood or serum, preferring an atmosphere containing about 10 percent carbon dioxide and a temperature of around  $35^{\circ}$  to

37° C. However a simplified medium for the gonococcus has been devised.<sup>4a</sup> They are also quite sensitive to low temperatures and drying. Meningococci have been found more resistant to drying than formerly supposed. They can live for days dried on coverslips. Sunlight and diffuse daylight are very destructive to them.<sup>4b</sup> These characteristics serve to differentiate them from the organisms described in the next paragraph and from *N. flavescens* and *N. catarrhalis*, which grow well on blood-free media at temperatures as low as 25° C. and are moderately resistant to drying and light.

The other seven species, best known of which are *Neisseria sicca* and *N. flava*, while parasites in a broad sense, being restricted to a life on a mammalian host, are usually quite harmless. As shown by

TABLE XIII  
DIFFERENTIATION OF NEISSERIA

Species	Glucose*	Sucrose*	Maltose*
<i>Neisseria gonorrhoeae</i> .....	+	—	—
<i>Neisseria intracellularis</i> .....	+	—	+
<i>Neisseria catarrhalis</i> .....	—	—	—
<i>Neisseria flavescens</i> .....	—	—	—
<i>Neisseria sicca</i> .....	+	+	+
<i>Neisseria flava</i> .....	+	—	+

\* These fermentation reactions are best determined on slants made by mixing 1.0 percent of the carbohydrates (already prepared in 20 percent aqueous sterile filtered solution) aseptically with infusion agar containing 20 percent ascitic fluid, and an indicator such as bromeresol purple.

Carpenter and others, however, under certain conditions some of them can cause serious infections which are sometimes confused with gonorrhea with tragic results.<sup>5, 6</sup> *N. flava* and *N. sicca*, as well as *N. intracellularis*, have been thus incriminated, so that no diagnosis of gonorrhea can be said to be complete and accurate without a full bacteriological study of the organism involved. This is especially true of institutional outbreaks of vulvovaginitis in little girls which, as shown by Nelson<sup>7</sup> and others, may sometimes consist entirely of infections due to *N. catarrhalis* or *N. sicca*. The *Neisseria* are all very similar, and may represent merely varieties of one species. Microscopically they are indistinguishable. The colored species produce colonies with greenish or yellow pigment, while the colonies of *N. sicca* are of a dry, crumbly consistency.

*Neisseria flavescens*<sup>8</sup> is a serologically homogeneous and distinct species and is easily distinguished from *N. gonorrhoeae* and *N. intracellularis* by agglutination tests, its yellow pigment, and fermentation tests. In many respects it resembles *N. catarrhalis*. *N. intracellularis* and *N. gonorrhoeae*, however, are much more closely related, being distinguished with difficulty by serological means and fermentation tests (see Table XIII). Indeed, *N. gonorrhoeae* has been found to cause meningitis while *N. intracellularis* has been isolated from conditions clinically indistinguishable from gonorrhea.

Cultivation of *N. gonorrhoeae* and *N. intracellularis* from pathological material requires special media and great care.<sup>9</sup> It is desir-

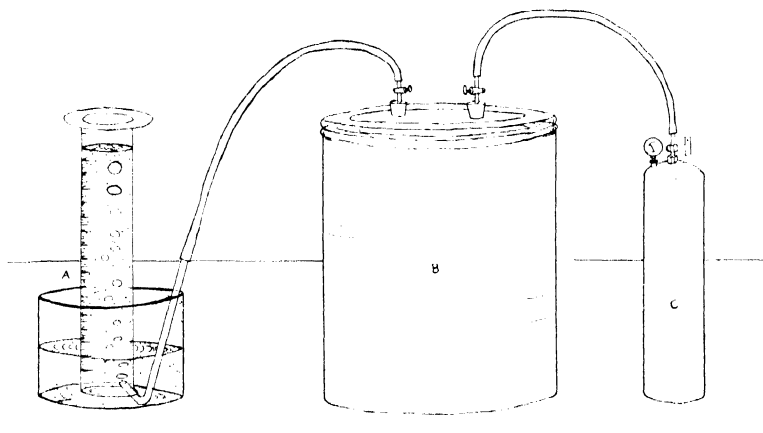


Fig. 295.—Diagram of method for providing an atmosphere with 10 percent  $\text{CO}_2$  for cultivation of various microaerophilic bacteria. The  $\text{CO}_2$  is admitted to the culture vessel B containing Petri dishes, tubes, etc., from the tank C. The displaced air is measured by water displacements in the graduated 2-liter cylinder A. The volume of B is measured previously.

able to use moist, freshly prepared, "chocolate" agar (add 5 percent blood to melted infusion agar at  $85^\circ\text{C}$ .—blood turns chocolate brown). The pus from gonorrheal infections, or sediment from spinal fluid, is streaked on the surface of the chocolate agar in plates, and the plates are then incubated in an atmosphere containing about 10 percent carbon dioxide (Fig. 295).

**Oxidase Reaction.**—The *Neisseria* possess a distinctive biological property which aids in their detection on the surface of blood-agar plates containing many different kinds of colonies

They produce an enzyme (oxidase) which causes a 1 percent solution of dimethyl-paraphenylene-diamine to turn, successively, pink, rose, magenta, and finally black (Fig. 296). The solution is flooded (1 cc.) over the surface of a 24-hour-old chocolate agar plate prepared as suggested above, and previously inoculated with, for instance, a swab from the vagina in an attempt to isolate gonococci. Colonies, which are at first colorless, translucent, flat and about 1 to 3 mm. in diameter, are fished as soon as the first rose tint is observed. These will grow in subcultures. If fishing is delayed too long, the cells are killed by the reagent. For subcultures



Fig. 296.—The oxidase reaction of *Neisseria*. Most of the colonies are oxidase-positive, having darkened the reagent applied to the plate. (Steinberg and Mollov, J. Lab. and Clin. Med., Vol. 27, C. V. Mosby Co.)

infusion agar slants with heated blood are best. For fermentation tests infusion agar containing 10 percent serum, 1 percent of the desired carbohydrate, and an indicator are used. The *Neisseria* seem to prefer solid media. Plates inoculated with swabs from the nasopharynx (the habitat of the meningococcus) often contain *N. sicca* and related species, all of which give the "oxidase reaction" and thus make the test inapplicable to such cultures if differentiation of the meningococcus is desired. Cultures from the genitalia rarely contain more than one type of *Neisseria*. If more than one type are found, all may be of diagnostic significance.

The *meningococci* are identified by, and separable into, four main types on the basis of agglutination reactions. These are types I, II, II<sub>a</sub> and IV. A "Quellung" reaction for "typing" is available, analogous to that used in typing pneumococci, since freshly isolated strains have been found to possess capsules. It has been used effectively by Cohen and others.<sup>9a</sup> As is the case with pneumococci and many other bacteria, these capsules contain immunologically specific soluble carbohydrates. It has been found by Phair and his associates that the serum of chickens immunized with meningococci of various types gives highly specific agglutination tests,<sup>10</sup> and the method has been used effectively in epidemiological studies.<sup>11</sup>

### PATHOGENIC ACTION OF THE NEISSERIA

Gonorrhea.—This is one of several diseases commonly spoken of as "venereal diseases," deriving this appellation from the name of Venus, goddess of love. The inappropriateness of this term will become obvious in the discussion of the infections.

Gonorrhea is an inflammatory disease due to infection by *N. gonorrhoeae*, of the mucous surfaces of the reproductive organs of both men and women. Much pus forms, and appears as a white discharge (leukorrhea) from the genitalia. It is an alarmingly prevalent disease, half a million cases or more being under medical care in the United States in 1944. There are undoubtedly many other, unreported and untreated cases.

Infection with *N. gonorrhoeae* occurs mainly through sexual intercourse, transmission by clothing or other fomites being so rare as to be negligible. Gonorrhea is seldom fatal but is sometimes difficult to cure. Patients often believe themselves cured only to find, later, that the disease has reappeared in a chronic form. The sulfonamid drugs have done much to rob gonorrhea of its terrors, but their use is attended with great danger unless controlled by a physician. Drug-fast strains are being found more and more frequently and indiscriminant use of the drugs may rob such therapy of its effectiveness because of adaptation to the drug by the gonococci.<sup>14</sup> Special tests are used to detect drug-fast strains.<sup>15</sup> Penicillin is used in treating infections with such strains.<sup>16</sup> It can be readily understood that careless, ignorant or malicious people can spread gonorrhea widely. Prostitution is one of the chief means by which the disease is propagated.<sup>12, 13</sup>

Gonorrhea, untreated, often results in sterility. The intense in-

inflammation caused by the endotoxins of the organism destroys the lining tissues of the genito-urinary tract, the tissue being replaced with scars. Such scars obstruct the *fallopian tubes* of the female and the *vas deferens* of the male (tubes through which the reproductive cells pass). Such scarred obstructions are called *strictures*. Stricture of the urethra in the male prevents urination and requires surgical intervention. Gonococci sometimes invade the body, localizing in the joints and the heart valves. In the former case, a very painful and stubborn type of arthritis (rheumatism, or inflammation of the joints) results while in the latter case a very damaging disease of the heart occurs, with permanent injury and sometimes death.



Fig. 296a. — The end-result of ophthalmia neonatorum due to the gonococcus. The person in attendance at birth failed to instill silver nitrate solution into this child's eyes. The mother had gonorrhea. (Courtesy Boston Nursery for Blind Babies.)

As pointed out in a previous section (sec page 610), disease of the genitalia, closely resembling gonorrhea due to the gonococcus, may be caused by certain species of *Neisseria* from the respiratory tract (*N. intracellularis*, *N. sicca*, etc.). These infections are nearly always entirely innocent but may result in tragic mistakes if not properly diagnosed.

*Gonorrheal Ophthalmia.*—An inflammation of the eye results when gonococci are rubbed into the eye. Loss of sight usually results unless treatment is prompt. A gonorrheal mother may infect her child at birth. Occasionally an adult with gonorrhea infects his own eyes through his own insanitation. Gonorrheal infection of the eyes of the newborn, or *ophthalmia neonatorum*, is one of the most important causes of blindness (Fig. 296a). Due to this fact most cities, states and countries require that physicians, nurses or midwives attending births, *regardless of any circumstances*, instill into the eyes of the infant a few drops of weak silver nitrate solution. This rapidly destroys gonococci. It is obtainable at any health department or drug store, in wax tubes ready for use, and is quite harmless.

*Laboratory Diagnosis of Gonorrhea.*—The diagnosis of *acute* gonorrheal infection in the adult male is usually based on microscopic examination of the pus. The pus is stained by Gram's method and the gonococci appear as gram-negative, coffee-bean-

shaped diplococci *within* the leukocytes (Fig. 298). Capsules can be demonstrated by staining with Wright's stain (Fig. 297).<sup>17</sup> In chronic cases, and in females, diagnosis by cultural methods as already described is much more certain and makes possible a distinction between infections due to *N. gonorrhoeae* and other organisms.

**Meningitis.**—The term meningitis is a pathological one and means, simply, inflammation of the membranes (meninges) covering the brain and spinal cord. It may be due to mechanical irritations, viruses, or any of several kinds of bacteria which may localize in the meninges. Among the bacteria are hemolytic strepto-



Fig. 297.—Capsule of *N. gonorrhoeae* stained with Wright's stain ( $\times 1000$ ). (Bernstein, Proc. Soc. Exp. Biol. and Med., Vol. 46.)



Fig. 298.—Meningococci in the leukocytes in the cerebrospinal fluid in a case of epidemic meningitis. The cocci occur in pairs and look like gonococci. The network between the cells is fibrin. (Jordan.)

cocci, tubercle bacilli, pneumococci, gonococci, influenza bacilli (*Hemophilus influenzae*) and occasionally a variety of other organisms, such as *Eberthella typhosa*, *Escherichia coli*, *Salmonella* species, and *Pseudomonas aeruginosa*. These are all more or less accidental infections, following usually upon extensive invasion of the blood stream after severe cases of infection of other parts of the body by the organisms involved. The meningococcus is the only common cause of epidemics of meningitis.

**Epidemic Meningitis.**—Meningitis is frequently due to the meningococcus. Carriers of meningococci are common, but meningitis is not.<sup>18</sup> There is evidence that the meningococcus very often causes conditions like rhinitis, "catarrh" or purulent "colds"

which heal and attract no particular attention because the etiological agent is unsuspected. It is supposed that only after it breaks down local tissue resistance at this site of localization does it invade the meninges and is probably carried there by the blood stream or along the nerve fibers leading directly from the nares into the brain. In many instances it proceeds first to the blood stream and may be cultivated on chocolate agar or in broth as described elsewhere. Very often it goes no further than the blood and never enters the meninges.

Once gaining entrance to the meninges the organism causes an intense, purulent inflammation of these tissues which is frequently fatal. The organism is transmitted in droplets of saliva by sneezing and coughing and, being so easily spread, often causes epidemics.

Epidemic meningitis due to the meningococcus is sometimes called spotted fever because of a rash. The organism can be cultivated from rash spots. It is also called *cerebrospinal fever*, or *cerebrospinal meningitis*. Crowded housing conditions are thought by some to favor the development of epidemics of meningitis due to *Neisseria intracellularis* because they favor transmission by coughing and sneezing. However, the evidence on this point is very confusing. A more important factor may be the state of immunity of the crowded population. The carrier state may quickly be cured with sulfonamid drugs.<sup>11, 19</sup>

*Laboratory Diagnosis of Meningitis.*—Diagnosis of meningococcus meningitis is made by examining gram-stained smears of the sediment in fluid from the spinal canal. The presence of the gram-negative, bean-shaped diplococci *within* the leukocytes suggests the diagnosis (Fig. 298). However, as stated before, gonococci and *N. flavescens* can also cause the disease and microscopic examination alone is not sufficient to establish the identity of the organism. The organisms may be cultivated on the same sort of media as are used for gonococci, but they must not be cooled at any time or exposed to strong light or allowed to dry after inoculation, since they are very sensitive to external conditions.

Blood cultures in warm, serum-dextrose infusion broth, made early in the disease, are frequently positive, a fact which lends support to the view that meningitis is often secondary to a blood-stream invasion.

Active artificial immunity (bacterin) in meningitis is of doubtful worth, and passive immunity (treatment with specific immune serum) is of relatively little value. If serum is used it should be administered early in the disease. As meningococci are of several



serological types, a so-called "polyvalent" serum (containing antibodies for the various types) is used.

Modern treatment centers about the use of sulfonamid drugs, which have reduced the case fatality rate from around 70 to 80 percent to something below 10 percent.

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## CHAPTER 39

### THE "LITTLE BACTERIA"

THE FAMILY Parvobacteriaceae ("little bacteria") was established to provide a convenient grouping for a number of bacteria which are relatively small ( $0.5\mu$  by  $3\mu$ ), gram-negative in staining, nonspore-forming, parasitic, aerobic rods. Some are motile, others nonmotile. Although, as thus described, they appear to resemble gram-negative rods common in the soil, they are distinctly not related to the soil or water or intestinal bacteria and seldom are found thriving in environmental habitats; *i.e.*, outside the human or animal body, except species (such as *Pasteurella pestis*) capable of existence in an insect host. As a group, they represent (especially the genera *Pasteurella* and *Hemophilus*) the highly parasitic type of bacteria requiring body proteins (blood serum or tissue) and "accessory growth substances" for their optimum growth. Further, they are rather sensitive to light, cooling, drying, etc., and lack the ruggedness necessary to growth and survival in the outer world which characterizes such organisms as the *Aerobacter*, *Serratia* and *Pseudomonas*. Generally speaking, they have little fermentative or proteolytic power, and do not cause notable changes in carbohydrate or protein media.

The family comprises some of the most dangerous pathogens such as *Pasteurella pestis* (the "plague bacillus"), *Past. tularensis* (the organism of "rabbit fever" or tularemia), *Malleomyces mallei* (the cause of glanders in horses and man), three species of *Brucella* (causing undulant fever in man and infectious abortion, etc., in animals) and *Hemophilus pertussis* (the cause of whooping cough).

There is a very extensive bacteriological literature concerning all of these organisms and an equally imposing medical literature about the diseases they cause. In the present discussion, only such of them and their properties will be described as serve to

illustrate special methods of cultivation or fundamental bacteriological or biological principles. *Past. pestis* is of interest historically.

**Genus *Pasteurella*.**—*Species Causing Hemorrhagic Septicemia.*—The genus is named for Pasteur, who founded the science of immunology on his studies of vaccination against *Past. avicida*, the cause of fowl "cholera." The bacteria of the group causing hemorrhagic septicemia are pathogenic for animals. The organisms invade the blood stream and may be cultivated on infusion media from all the organs. There are many small hemorrhages on various internal mucous surfaces, on the skin, and in the internal organs, hence the name "hemorrhagic septicemia." There is much exudation of fluid from nose, mouth, eyes, etc. This fluid is highly infec-

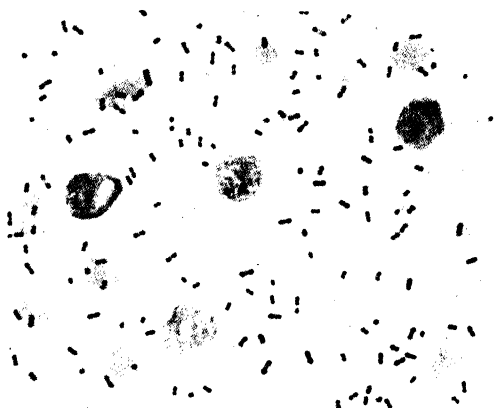


Fig. 299.—*Pasteurella bovisseptica* from heart blood of infected rabbit, stained with methylene blue ( $\times 900$ ). (Ford.)

tious and transmits the disease. Among the animals affected are birds, notably domestic fowl (fowl cholera), rodents, swine, sheep, and cattle. The disease is rapidly fatal as a rule.

Several species of organism causing hemorrhagic septicemia are known, although the distinction between some of them is exceedingly slight and of doubtful significance. For example, the organism causing the disease in chickens is called *Pasteurella avicida*; that causing swine plague is called *Past. suilla*; that causing hemorrhagic septicemia in rabbits is called *Past. cuniculicida*, and so on. However, *Past. avicida* can infect rabbits and *Past. suilla* can infect birds and rodents, and, in general the species seem to be interchangeable to a great extent.

Culturally, the organisms are not refractory, growing well on infusion agar or serum agar and in ordinary infusion broth. They are not very active biochemically, neither fermenting carbohydrates nor hydrolyzing gelatin.

The morphology of the organisms is rather characteristic. In pathological material they are short, oval rods, about 1 micron by 6 microns in diameter and length, which tend to stain most heavily at the tips. This gives them a distinctive appearance (bipolar staining, Fig. 299). In culture media the bipolar appearance is less definite. They are not motile. They succumb readily to heat and disinfectants but are often the cause of severe and costly

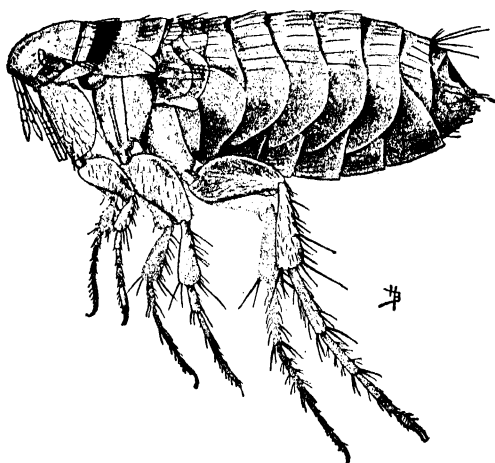


Fig. 300.—The European rat flea (*Ceratophyllus fasciatus*): Adult female, greatly enlarged. (Bishopp: Bull. 248, U. S. Dept. of Agriculture.)

epidemics among laboratory and farm animals. These epidemics can be controlled only by rigid isolation, disinfection, and killing sick animals.

***Pasteurella pestis*.**—From the standpoint of mankind, the most important member of the genus is *Past. pestis*, the cause of bubonic and pneumonic plague in man.<sup>1</sup> Morphologically and culturally, *Past. pestis* resembles the other members of the genus closely but is slower and less vigorous in its growth.

**Bubonic Plague.**—Bubonic plague, due to *Pasteurella pestis*, is an outstanding example of a bacterial disease transmitted by the bite of an insect. It is conveyed to human beings by the bite

of infected fleas (Fig. 300), most important of which, in this respect, are *Xenopsylla cheopis* and *Ceratophyllus fasciatus*, the rat fleas. The insects usually derive the plague bacilli from the blood of the rats *Mus norvegicus* and *Mus rattus* which may be infected and which often show lesions similar to those in man (see p. 624). The former species of rat inhabits especially ships, sewers, stables and city refuse dumps. The latter is principally a house rat. Rats have been shown to maintain plague as an epizootic among themselves for long periods and to act, therefore, as an *animal reservoir* of plague bacilli. When rats become excessively prevalent in any locality, human plague is apt to occur because the opportunity for rat fleas to bite human beings greatly increases. Populations living near dumps or in dirty unsanitary conditions suffer most. Conditions following the devastation of war, with breakdown of disease-control systems, are ideal for the development of rats and, therefore, rat-borne diseases.<sup>7</sup> The pages of history are filled with disasters to armies and civil populations attacked by plague. The trouble with "General Rat" and "General Flea" as military allies is that they fight on both sides. The rats often die in great numbers from the disease and the fleas tend to leave the cooling bodies, jumping on to the first warm animal which passes. Dead or dying rats, therefore, are potentially dangerous. Further, it has been shown that plague bacilli can survive for months in the bodies of dead animals so that these should not be handled carelessly if plague is suspected.

Since 1900, plague has been found in the United States in rodents other than rats, especially ground squirrels or "prairie dogs." Many human cases have been traced to wild rodents. The disease in woodland or wild-living creatures is spoken of as sylvatic plague.<sup>2-6</sup> The control of wild rodents is a field problem of great importance.

Plague is traditionally a disease of travel routes and, in rats, accompanies caravans, armies, and ships to foreign places. The United States Public Health Service frequently institutes "rat surveys" of seaports in the United States with the object (a) of determining the prevalence of rats, and (b) of determining the percentage of rats infected. The work is hazardous and should not be indulged in by amateur bacteriologists. The rats are trapped and inspected for infected fleas or for lesions of the disease,\* or both. When either factor reaches too high a level, anti-rat campaigns are

\* Principally enlarged lymph glands (buboes). Smears and cultures may show the bacilli.

instituted. Traps are set, garbage dumps are cleaned, buildings are "rat-proofed" and numerous other precautions are taken. The ropes used to moor ships from foreign countries to wharves are always required to be guarded by means of large metal disks for the purpose of preventing rats from coming ashore. The rats often circumvent this, however, by dropping into the water and swimming ashore, or by climbing over the guard (Fig. 301).

**Plague in London, 1665.**—In rat-infested cities plague may spread with great speed and reach terrifying levels of morbidity and fatality. The disease has been known as a frightful scourge

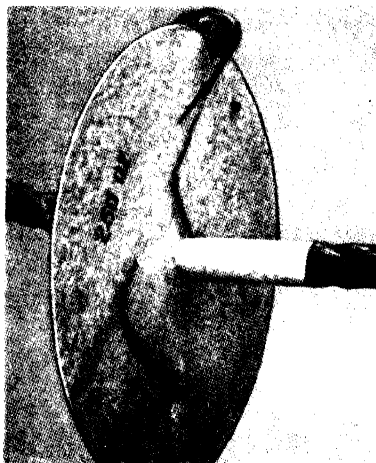


Fig. 301.—Rat climbing over guard placed on ship's mooring line. (Denney, O. E., U. S. P. H. S., Weekly Reports.)

since biblical times. In the two years between 1347 and 1349 it is thought to have killed one-fourth of the whole population of Europe, or about twenty-five million people. Known as the "black death" (because of the dark hemorrhagic spots), it was indeed, as stated by Holmes,<sup>8</sup> one of the shadows of the Dark Ages. One of the most famous epidemics of bubonic plague was that which occurred in London in 1665, and which was later described by Daniel Defoe in his "Journal of the Plague Year." It is probable that Defoe did not actually observe the things he described, but his pictures are based on facts. Defoe

was a nonscientific writer and at that time neither the cause nor mode of transmission of plague was known. Mysticism and witchcraft were still believed in. Some idea of the intensity of the epidemic may be gained from a statement by Defoe:

"... there was no parish in or about London where it raged with such violence as in the two parishes of Aldgate and Whitechapel.

"They had dug several pits in another ground, into these pits they had put perhaps fifty or sixty bodies each; then they made larger holes wherein they buried all that the cart brought in a week, which by the middle to the end of August, came to from two hundred to four hundred a week; and they could not well dig

them larger, because of the order of the magistrates, confining them to leave no bodies within six feet of the surface; and the water coming on at about seventeen or eighteen feet, they could not well, I say, put more in one pit; but now, at the beginning of September, the plague raging in a dreadful manner, and the number of burials in our parish increasing to more than was ever buried in any parish about London, of no larger extent, they ordered this dreadful gulf to be dug, for such it was rather than a pit.

"... the pit being finished the fourth of September, I think they began to bury in it the sixth, and by the twentieth, which was just two weeks, they had thrown into it eleven hundred and fourteen



Fig. 302.—*Pasteurella pestis* in pus from the site of a flea bite on the ankle of the sick woman mentioned in Fig. 303. Note the bipolar staining of the oval rods.

bodies, when they were obliged to fill it up, the bodies being then come to lie within six feet of the surface.

"... and this was the time that the bills (lists of dead) came up to such a monstrous height, as that I mentioned before; and that eight or nine, and as I believe, ten or twelve thousand a week died; for it is my settled opinion, that they never could come at any just account of the numbers, for the reasons which I have given already.

"... it pleased God, with a most agreeable surprise, to cause the fury of it to abate, even of itself, and the malignity declining, as I have said, though infinite numbers were sick, yet fewer died; and the very first week's bill decreased eighteen hundred and forty-three—a vast number indeed!"

**Plague in Man.**—The disease in human beings is characterized by the usual symptoms of an infection and often by a pustule developing at the site of the infecting bite. This is not always found. The pus teems with plague bacilli (Fig. 302). The bacteria travel along the blood and lymph vessels and are taken up by the nearest lymph glands situated in the groin, arm pits, neck, etc., where secondary foci of infection occur, the glands becoming greatly enlarged. They are then called *buboes* (hence *bubonic*) and are filled with a “puree” or thick pus teeming with bacilli. These



Fig. 303.—A plague-infested town in Brazil. A woman lay dying in the house at the extreme right; two girls were recovering with draining buboes, in the house at the extreme left, while a boy and girl (seen in the windows) had fully recovered and had scars in the groin, in the house where the man is entering. Two dead rats were found in the gully in the center foreground. The bacilli seen in Fig. 302 were obtained from the dying woman.

buboes often break open and discharge their contents and may heal, leaving scars. On the other hand, death may occur in a few days. Material taken from the buboes, or from the blood, may be smeared, cultured, or injected into guinea pigs in which the organisms cause a bubonic and hemorrhagic disease similar to that seen in rats and man.

Today, human plague is rare in North America but is found in the Orient, some cities of South America (Fig. 303) and Central Europe. In the middle ages plague was so common that a painting of St. Roch at Lugano shows a plague bubo on the thigh (Fig. 304).



While the mortality is high, obviously plague is not always fatal and varies greatly in this respect in different epidemics. The pneumonic form of plague is particularly fatal, and is transmitted by coughing and sneezing. It develops when the organisms localize in the lung tissues from other sources in the body, and are then excreted to the exterior in the pus and mucus of the sputum.

Prevention of plague obviously depends chiefly upon suppression of rats and this in turn is based on good municipal sanitation and



Fig. 304.—Painting of St. Roch with plague bubo on thigh.

avoidance of conditions favorable to rat multiplication. Vaccines of killed bacilli are said to be effective but only to a limited extent. They reduce *mortality*, but not *morbidity*.

***Pasteurella tularensis*.**—This member of the genus is much like *Past. pestis* in most respects, being a small, nonmotile, gram-negative, aerobic, nonspore-forming rod with a tendency to become rounded and to stain like *Past. pestis*. It is more exacting in its nutritional requirements than the latter. Growth is much enhanced by the amino acid cystine or (what amounts to the same

thing) by media containing compounds with the -SH (sulfhydryl) group.

On ordinary blood agar it rarely grows on first isolation, but if a small amount of cystine be added, growth will usually occur. Coag-



Fig. 305.—A Primary ulcer on the temple following bite of fly, *Chrysops discalis*, in Utah. (Dr. Francis, Army Medical Museum, No. 42955.) B Primary ulcer on finger following dressing of wild rabbits; eighteen days after onset. (Dr. Tomas Cajigas, Army Medical Museum, No. 63174.) C Ulcer of thumb and axillary bubo after dressing market rabbits; forty days after onset of illness. (Drs. Brown and Hunter, Army Medical Museum, No. 43709.)

ulated whole egg, which contains cystine or at least compounds with the sulfhydryl group, is a superior medium. Bits of rabbit spleen rubbed over the surface of agar media also aid growth on the agar.

*Past. tularensis* is found in much the same ecological relationship to rodents (rabbits, gophers, mice), biting insects (wood, dog, and rabbit ticks, rabbit lice, deer-flies, horse flies) and man as is *Past. pestis*. It causes a disease called tularemia.

*Tularemia* ("Rabbit Fever").—This disease is common in many American rodents and other animals, in which it causes a plaguelike disease, with glandular enlargements, swelling of the spleen and the appearance of whitish nodules in the liver, spleen and elsewhere. These somewhat resemble tubercles in gross appearance. The bacilli invade the blood stream from these foci just as *Past. pestis* does in plague, and thus biting insects, especially various ticks, flies and rabbit lice, may transmit the organisms from one animal to another and to man. The disease in man is analogous to that in animals, but is much less likely to end fatally.

One common means of transmission is by handling infected rabbits, as in the marketing of rabbits for food and pelts.<sup>9, 10</sup> The initial lesion, an ulcer or "sore," analogous to the pustule of plague flea bites, is seen usually on the hands or face but may not be recognized until general symptoms of fever, pains and glandular enlargements become prominent (Fig. 305). The organism can infect by almost any route. Agglutination tests become positive after subsidence of the early phase of the disease.

**Genus *Brucella*.**—*Brucella melitensis* and two closely related species, *Br. abortus* and *Br. suis* cause the disease *Malta fever* or *undulant fever*, common here as well as in many other parts of the world. The generic name is derived from the discoverer, Bruce, a British scientist who first (1887) found the organism now called *Brucella melitensis* on the island of Malta in the spleens of persons infected by the organisms in goat's milk. The organism found by Bruce was called *M. melitensis*, Malta having, in ancient days, been known as Melita because of the fine honey found there. Because of the frequent occurrence of very short, coccoid forms, Bruce first thought his organism to be a micrococcus.

*Br. abortus* was first known as a bacillus causing abortion in animals. It was discovered by a Danish worker named Bang in 1895 and the organism is still often referred to as Bang's bacillus.

*Br. suis* was first observed by Traum in the United States in 1914. Its true relationship to undulant fever was not then known. Only in 1918 did the close relationship of these three organisms become clear through the work of Evans in Washington, D. C.<sup>12</sup>

The organisms of all of the above species are small, gram-negative, nonmotile, nonencapsulated, nonspore-forming rods tending

to become coccoid (Fig. 306), growing poorly when first isolated from the milk or from the blood or tissues of infected animals or man, and best cultivated in slightly acid (*pH* 6.8) liver-infusion agar or liver broth.\* *Brucella abortus* will grow well, at first, only in an atmosphere containing 10 percent carbon dioxide. This peculiarity is shared with a number of other pathogens, especially the *Neisseria*. No carbohydrates are fermented, but

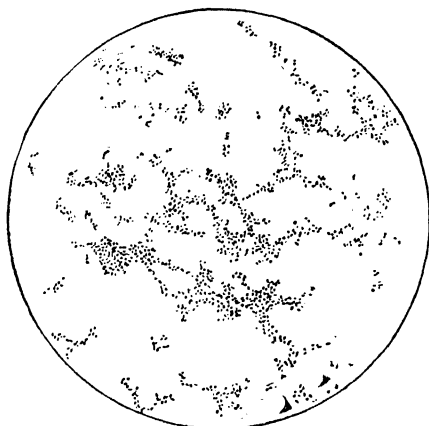


Fig. 306.—*Brucella melitensis* ( $\times 900$ ). (Mohler and Traum.) Note the very small size and coccoid appearance of the organisms. In some cultures they appear larger.

they appear to be metabolized with alkaline end-products. All species are somewhat microaerophilic.

• **Liver-infusion agar** (Adapted from Huddleson, I F., "Brucellosis in Man and Animals," The Commonwealth Fund, N. Y., 1939):

Obtain fat-free liver and grind 1 pound to a plastic mass.

To 500 cc. add 500 cc. of distilled  $H_2O$  and infuse for 24 hours.

Steam 20 minutes in Arnold, stir, and continue heating for  $1\frac{1}{2}$  hours. Filter through a wire screen.

For 1 liter of liver-infusion agar mix:

Agar.....	20 gm.
$H_2O$ (distilled).....	500 cc.
Infusion (prepared as above).....	500 cc.
Peptone.....	5 gm.
NaCl.....	5 gm.

Heat at  $100^\circ C$ . for 1 hour, then cool to  $60^\circ C$ . and adjust *pH* to 7.2 (will drop to about 6.8 on further heating).

Heat at  $100^\circ C$ . for 30 minutes; decant, dispense and autoclave.

The agar medium need not be clear.

The broth is prepared in the same manner, with omission of the agar, and filtration through paper after the second heating. Use only when freshly prepared.

The three organisms in this genus are closely similar but may be differentiated according to the animal they most commonly infect. As shown by Huddleson<sup>11</sup> and others, they may also be distinguished, sometimes, by the characters given in Table XIV.

TABLE XIV  
CHARACTERS USED TO DIFFERENTIATE SPECIES OF BRUCELLA

Species	Number of days from Commencement of Growth during Which H <sub>2</sub> S is Continuously produced.	Utilization (Not Fermentation) of Dextrose.	Degree of Growth Vigor on Liver-Infusion Agar Containing 1 part in 25,000 of	
			Thionin	Basic Fuchsin
<i>Brucella melitensis</i> ....	No H <sub>2</sub> S	active	good	good
<i>Brucella abortus</i> .....	4	slight	poor	good
<i>Brucella suis</i> .....	6-10	active	good	poor

Agglutination tests are of little value in differentiation of these species from one another.



Fig. 307.—*Brucella bronchiseptica* (× 900).

One other organism of this genus, *Br. bronchiseptica*, is a good illustration of the difficulty of classifying bacteria (Fig. 307). In its nonfermentation of dextrose and nonliquefaction of gelatin it

resembles the brucellas but in many other respects it is also almost identical with *Alcaligenes faecalis*. It is also motile. It has certain factors in common with the organism of whooping cough (*Hemophilus pertussis*) described later in this chapter and it will be discussed there, although it is very different in many other important characteristics. *Br. bronchiseptica* is not related to undulant fever.

**Undulant Fever (Brucellosis).**—This disease is caused by members of the genus *Brucella*. Undulant fever in man is characterized by a long, slow onset with increasing weakness and lassitude. Later there is backache, stiffness of joints, anorexia (loss of appetite), loss of weight, low fever and many other indefinite symptoms, depending on the organs affected by the bacteria. Remittent attacks of fever of a day's or several days' duration, with severe night sweats, persist over long periods. Rheumatic symptoms are common. Sometimes mild attacks of the disease are diagnosed as influenza or "grippe." The disease is seldom fatal.

Like *Pasteurella tularensis*, the bacilli invade the blood stream and localize in foci in the lymphatic system everywhere, but especially in the spleen and liver. They may occur in urine and feces and are fairly resistant to the conditions of the outer world. In cows and milch goats they tend to localize in the udder and appear in the milk. The organisms are transmitted from animals (cattle, swine, goats) to each other and to man by means of infectious discharges, milk or carcasses. For this reason the disease is found most frequently among farmers, slaughter-house employees and persons in districts where the disease is common in dairy cattle and who drink unpasteurized, uncertified milk. The organisms enter the body from the sources mentioned by almost any portal of entry, but particularly through abrasions of the skin and through the gastro-intestinal tract.

*Brucella abortus* derives its name from the fact that it causes, chiefly, infectious abortion in cattle. Being readily transmitted to man and from cow to cow by milk, vaginal discharges, possibly dung and saliva, and perhaps by other animals, it is difficult to control and may cause great economic loss. It occurs frequently in human disease in this country. *Br. melitensis* causes little disease because the use of goats' milk is uncommon here. *Br. suis* causes the most severe disease in man and is commonest among those raising hogs and handling pork products. The swine species (*suis*) may infect not only swine and human beings, but goats or cattle, while the goat species (*melitensis*) and the bovine species (*abortus*) may infect man and other animals.<sup>12-16</sup>

**Laboratory Diagnosis.**—The brucellas readily infect guinea pigs if injected into them, causing a disease chiefly of the lymphatics, with nodules and bubo-like enlargements suggestive of tubercles or of the nodules caused by the *Pasteurella* group. Guinea pig injection is, therefore, often used in diagnosis or in attempts to isolate the organisms from blood, or cream, and is sometimes more satisfactory than direct cultivation. However, persons handling infected guinea pigs almost invariably contract the disease, which often disables them for long periods.

If cultures are used, liver broth is best for blood while, for cultivation from cream, plates of liver agar with a selectively inhibitory dye (crystal violet, 1:500,000) to restrain *Streptococcus lactis* and other gram-positive organisms, are sometimes successful. Requirement of *Br. abortus* for carbon dioxide must be remembered. As growth of the brucellas is slow the cultures should be incubated for two weeks unless colonies appear earlier.

The *agglutination test* is a good means of detecting the presence of brucellosis in cattle and in man, especially if a series of tests are made with serum specimens taken at intervals of a week or so after onset of fever, and a distinct rise in titer is noted. The tests may also be made with rennet-whey of cows' milk. Herds of dairy and breeding cattle are often examined by means of the agglutination test (often spoken of "Bang testing") to detect latent infection. Low titers (serum diluted to around 1:20 or 1:30) are of doubtful significance but high titers (serum dilutions of from 1:100 to 1:1000 or more) indicate infected udders or probable abortion if the cow is pregnant. The opsonocytophagic test, an evaluation of the ability of leukocytes to ingest the bacilli in the body, is a useful additional method of diagnosing infection or detecting possible susceptibility.<sup>17</sup>

**Genus Hemophilus.**—This genus of the family Parvobacteriaceae contains some very interesting illustrations—not only of nutritional fastidiousness which emphasizes the metabolic limitations of highly parasitic organisms, but also a pathological relationship of fundamental importance. There are several species in the genus, important among which are the "influenza bacillus" (*Hemophilus influenzae*, Fig. 308), the organism of whooping cough (*H. pertussis*), *H. suis*, implicated in swine influenza, and *H. ducreyi*, the cause of a venereal disease called "soft chancre."

The general description of the family fits these organisms perfectly since they are gram-negative, nonspore-forming, aerobic and facultative, very tiny, nonmotile, highly parasitic and sensi-

tive to all but body conditions. These organisms do not, as a rule, ferment dextrose and they are not proteolytic.

*Special Nutritive Requirements of the Hemophilic Organisms.*—Their generic name is derived from the fact that they require blood, or certain substances in it, for satisfactory growth, especially when freshly isolated from the body. One substance, for years unknown and therefore called an "X factor," is now recognized as the iron complex called *hematin*, which occurs in the red coloring matter of erythrocytes. This probably plays some role in their respiratory functions since, as pointed out previously (see page 369), iron can exist in both oxidized and reduced form and therefore constitutes an oxidation-reduction system.



Fig. 308.—*Hemophilus influenzae* ( $\times 900$ ).

In addition, most of the species require for growth a formerly unknown substance, originally referred to as "V factor" but now recognized as coenzyme I, extractable from yeast and certain other vegetable cells and also from some bacteria, notably certain strains of staphylococci. The X factor withstands moderate autoclaving, the V factor is easily destroyed by heating. Fresh, whole blood apparently contains both X and V factors in sufficient amounts for hemophilic bacteria. The relationships of these factors to growth of some of the organisms is shown in Table XV (see also section on bacterial respiration, page 365). However, it is possible to cultivate *H. pertussis* on media without blood.<sup>18</sup>

Fresh blood, especially human blood, also contains some in-



hibitory substance which may be removed by heating for ten minutes at 90° C. The blood turns a chocolate brown and, in agar, is referred to as "chocolate agar." It is useful for many pathogens, especially the genus *Neisseria*.

"*Satellite Formation*."—A striking demonstration of the synthesis of coenzyme by staphylococci is seen when a plate containing over-heated blood agar (minus the V factor) is inoculated with *H. influenzae* all over the surface and in one spot only with an appropriate strain of *Staphylococcus*. Upon incubation, growth of *H. influenzae* is found to have occurred only in the immediate neighborhood of the *Staphylococcus* colonies (Fig. 309).

TABLE XV

RELATIONSHIP OF "X" AND "V" FACTORS TO GROWTH OF HEMOPHILUS

Growth in Peptone Broth or Agar	<i>H. influ- enzae</i>	<i>H. suis</i>	<i>H. pertussis</i>	
			Fresh*	Old†
With neither factor .....	—	—	—	+
With X factor only .....	—	—	—	++
With V factor only .....	—	—	—	++
With X and V factors .....	+	+	+	++
With whole blood .....	+	+	+	++

\* First isolation from the throat (Phase I).

† After several transfers on artificial medium (Phases II, III, IV).

**Hemophilus influenzae.**—The pathological relationships of this organism are interesting. It derives its name from the fact that it was formerly thought to cause influenza or colds. It is commonly found in the normal nose and throat. The consensus of opinion today is that it has no such relation though it may augment the severity of colds and influenza by infecting the already diseased nasal mucosa. It causes a very severe type of laryngotracheitis in infants. It is often found alone in diseased lungs and in cases of meningitis, which it apparently may cause,<sup>19</sup> and in cases of endocarditis (disease of the heart valves). It grows well on ordinary rabbit blood agar plates; the colonies are 0.5 to 1 mm. in diameter, colorless, and transparent like flattened dewdrops. The colonies are seen with difficulty. *H. influenzae*, like some other bacteria, undergoes the R-S variations. In the R phase it produces very long filaments and swollen cells and may look quite unlike the traditional

type of "little bacteria." Some of these filaments are seen in Figure 308. This sort of morphological variation is seen in many bacteria.

Six serological types (called a, b, c, d, e and f) of influenza bacilli are known, as well as several variants which differ in respect to such activities as indol formation, intrate reduction and hemolysis production in blood agar plates. One variant is often called *H. hemolyticus*. It is common in normal throats and the colonies are sometimes mistaken for those of beta-type hemolytic streptococci.

The type b influenza bacillus has recently come into prominence as the cause of severe throat infections especially in young chil-

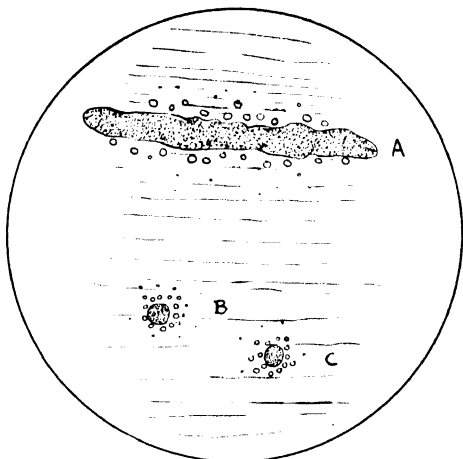


Fig. 309.—Diagram of "satellite" formation by *Hemophilus influenzae* on overheated "chocolate-agar" plate. The entire plate was streaked with *H. influenzae* culture and then inoculated at A, B and C with *Staphylococcus aureus*. Upon incubation, luxuriant growth of staphylococci occurred at A, B and C, but *H. influenzae* colonies (the small circles) developed only in the immediate vicinity of the *Staphylococcus* growth, where the V factor, absent from the overheated agar, is given off by the *Staphylococcus* cells.

dren. The organisms may be isolated on rabbit blood agar from sputum and, in some cases, from the blood. The type of bacilli causing the infections is determined by means of a capsule-swelling reaction analogous in all respects to that used for typing pneumococci and meningococci (see pneumococcus typing, page 604). The capsule appears to consist of a toxic specific carbohydrate and bears much the same relation to the pathogenesis of the infections as the capsules of *D. pneumoniae* (see page 606). The use of type-specific immune rabbit serum has been proven highly effective in diagnosis and therapy, by Alexander, and others.<sup>20</sup>

**Hemophilus pertussis.**—This, the bacterium causing whooping cough (pertussis) (Fig. 310), was discovered in 1906 by two Belgian workers, Bordet and Gengou. It is sometimes called the Bordet-Gengou bacillus. It is best cultivated from the body on agar containing glycerin, potato extract and blood (Bordet-



Fig. 310.—Electron microscope photograph of *Hemophilus pertussis* ( $\times 50,000$ ). Actually, the bacterium that causes whooping cough is about 0.5 micron in length; this is approximately equivalent to 0.0000195 of an inch. The bacterial body shown is enveloped by a "membrane" or capsule which shows as a lighter surrounding area. The capsule of *H. pertussis* is antigenic. (Dr. Malcolm H. Soule in Therapeutic Notes, May-June, 1943, Parke, Davis & Company.)

Gengou medium)\* seeming, when first isolated, to require large amounts of both X and V factors. The organisms are coughed up in

\* **Bordet-Gengou medium** (*Kendrick's modification*):

Peeled sliced potatoes.....	500 gm
Glycerin, U.S.P.....	40 cc.
Dist. H <sub>2</sub> O.....	1000 cc.

Boil the potatoes in the glycerin and water until soft. Replace water lost by

great numbers during the first three or four weeks of the disease. They may be caught and cultivated on Petri plates containing



Fig. 311.—Use of the cough plate in the diagnosis of pertussis. The Petri dish contains Bordet-Gengou medium and the child is coughing across it. It will be closed and incubated. From cultures such as this Phase I cultures are obtained for the preparation of pertussis vaccines. Photograph by Dr. Franklin H. Top. (*Therapeutic Notes*, May-June, 1943, Parke, Davis & Company.)

evaporation. Pass the fluid portion through gauze and allow sediment to settle out. Decant the supernatant liquid.

To 500 cc. of this fluid add:

NaCl (0.75 percent solution) .....	1500 cc.
Agar .....	60 gm.
Proteose peptone .....	20 gm.

Heat until agar is dissolved, make up to volume, and dispense in desired amounts for storage. Autoclave at 15 pounds' pressure (120° C.) for 25 minutes. When needed, this agar is melted, cooled to 45° C. and blood is added to make a final concentration of at least 15 percent. Defibrinated blood should be used, which is not more than 72 hours old. Mix blood and agar and pour plates fairly thick (15–20 cc.).

Bordet-Gengou agar held in front of the victim's mouth during paroxysms of coughing ("cough plates") (Fig. 311). The colonies appear only after ninety-six hours at 37° C. and look like very tiny pearls or droplets of mercury. On the garnet-colored medium, they are surrounded by a dark, changed zone of hemolysis (Fig. 312). Cough plates are very widely used in the bacteriological diagnosis of pertussis. Nasopharyngeal swabs are also used with excellent results in collecting cultures.<sup>21</sup>

*Phases of H. pertussis.*—When freshly isolated the organisms are encapsulated,<sup>22</sup> virulent for guinea pigs, mice and rabbits, hemolytic and very fastidious about X and V factors. This is spoken of as Phase I of the organism. After artificial cultivation it loses these properties and degenerates to Phases II, III and IV. It is important to note the development of these phases as one aspect of the general problem of bacterial variation which has a direct bearing on health.

Phase I strains of the pertussis organism, killed by appropriate means, when injected into the body stimulate the production of protective antibodies and thus serve as a means of preventing or modifying the severity of whooping cough.<sup>23</sup> If older strains of Phases II, III, or IV, maintained for some time on blood-free media, are used, no protective stimulation is obtained. Before these facts were known, many failures occurred in attempts to vaccinate against pertussis. This is illustrative of similar variations in other species of bacteria, notably *E. typhosa*, in which Vi strains of the smooth, virulent type are used for bacterins, degenerate rough or Vi-free strains having little or no virtue as bacterins.

**Whooping Cough.**—The principal clinical manifestations of whooping cough, or pertussis (due to *Hemophilus pertussis*) are so well known that no description need be given. The disease is common enough in children, usually becoming epidemic in the early spring although winter and fall outbreaks are not uncommon. In older children it is disagreeable but not excessively dangerous.

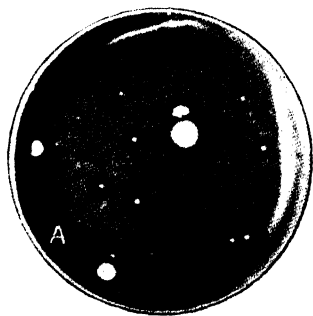


Fig. 312.—"Cough plate" from child with pertussis. On Plate A the colonies of *Hemophilus pertussis* are very tiny and are surrounded by zones of hemolysis. The large colonies are probably staphylococci. (Sauer and Hambrecht.)

In children under three years of age it is one of the principal forerunners of fatal pneumococcus pneumonia and thousands of children die from this cause annually in the United States.

Pertussis begins like an ordinary cold, the cough being worse at night. It is most infectious during the first week or two after onset. Transmission is, of course, by means of droplets of saliva and nasal and oral mucus. Infectivity ceases after the sixth week from onset because of the disappearance, at this time, of the bacilli from the trachea of children having the disease. Hence, cases may safely be released from quarantine after the sixth week following onset but there is really little danger after four weeks. The patient develops a life-long immunity.

The cough in pertussis may



Fig. 313.—Whooping cough. Minute bacilli present in masses between cilia of two cells lining the trachea: in the lower part of the picture are seen the nuclei of three epithelial cells of the tracheal lining. Above the nuclei is the cytoplasm of the cells, and on the surface are masses of *Hemophilus pertussis*.  $\times$  about 1500. (Mallory and Horner.)

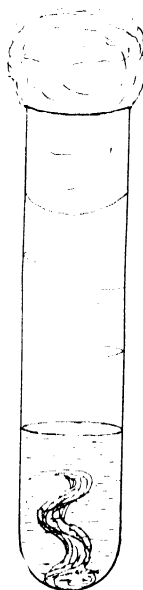


Fig. 314.—Culture of *Hemophilus pertussis* (Phase IV) showing slimy or "ropy" sediment in broth.

be due to a toxin which has been demonstrated by Wood<sup>24</sup> and others<sup>25</sup> to be present in the pertussis bacilli and to appear in filtrates of their cultures. The organisms gain a foothold in the trachea and grow among the cilia of the tracheal epithelial cells, setting up an irritation there (Fig. 313). They may be enabled to retain their footing by means of a slimy secretion which is often seen in broth cultures of certain phases (Fig. 314). The relation of this slimy substance to the capsule is still obscure.

Passive immunity may be of some value,<sup>26</sup> but the use of bacterins made of Phase I strains seems to be of greatest usefulness in the prevention or in lessening the severity of the disease.

**Hemophilus suis** is almost identical with *H. influenzae*, differing from typical strains of the latter mainly in failing to produce indol. It is associated with swine influenza, but the relationship is complex. By itself, it causes no disease in swine. Swine influenza is due to a filtrable virus (see page 727). The disease as caused by the virus alone is relatively mild, often passes unnoticed and is seldom fatal. If, as usually occurs under natural conditions, the bacterium happens to accompany the virus when passed in the nasal and oral secretions from one pig to another, the disease caused is often fatal.



Fig. 315.—Bacillus of Ducrey (*Streptobacillus*), the organism that causes soft chancre or chancroid. ( $\times 900$ .) Note the "schools." (Drawn from Kolle and Wassermann.)

This illustrates a principle of the greatest importance. It may be found that many other diseases of obscure etiology represent combined infections of a virus and a bacterium. One long-known example is that bane of the farmer, hog cholera. This disease is primarily one of the blood, is due to a virus and in the uncomplicated form is not excessively fatal. With the added infection of *Salmonella choleraesuis* or *Pasteurella suilla*, it becomes a problem of great economic importance, as the intestinal and lung involvement greatly increases the loss of swine. Unlike swine influenza, the bacterial component of hog cholera produces a dangerous infection by itself in both man and animals.

**Hemophilus ducreyi**.—This organism is physiologically closely

related to *H. influenzae* and *H. pertussis* and is morphologically very much like them. It is not found in the respiratory tract but may be cultivated from the lesions of a venereal disease called "chancroid" or "soft chancre" (as contrasted with the hard chancre, or ulcer, of syphilis). It grows best on soft blood agar<sup>27</sup> or heated, clotted blood and tends to form long filaments, which, in pathological material, are characteristically seen in clumps or "schools" (Fig. 315).

It causes a spreading, and often very destructive, ulceration and gangrene of the external genitalia and adjacent parts and may be seen in smears of pus from such lesions. It is transmitted chiefly by sexual intercourse. Like other members of the genus, it is a fragile organism and soon dies outside the body.

***Brucella bronchiseptica*.**—As shown by Brown<sup>28</sup> and others, this organism occasionally causes a pertussis-like disease of children. It

TABLE XVI  
CULTURAL CHARACTERS DIFFERENTIATING *BACILLUS PARAPERTUSSIS* FROM  
RELATED BACTERIA

	NO <sub>2</sub> Reduction	Growth on Plain Agar	Growth in Plain Broth	Motility
<i>Hemophilus pertussis</i> (Phase I).....	—	—	—	—
<i>Bacillus parapertussis</i> .....	—	++ (brown pigment)	++ (ropy; brown pigment)	—
<i>Brucella bronchiseptica</i> .....	+	++	++	+

is a common cause of respiratory disease ("snuffles") of rabbits and other small animals. Unlike *Hemophilus pertussis*, it grows readily on ordinary agar. It should be classed in the genus *Alcaligenes*. In several other respects it resembles *H. pertussis*, being a gram-negative rod, nonspore-forming and nonencapsulated. However, it is actively motile, much larger and grows much more vigorously and is more resistant to unfavorable influences. In these respects it resembles the intestinal organisms. It may be identified by agglutination tests and by other characters shown in Table XVI.

Eldering and Kendrick<sup>29</sup> described a closely related organism which they named *Bacillus parapertussis*. It has properties of both *H. pertussis* and *Br. bronchiseptica* (Table XVI). It also is not infrequently found in cases of pertussis-like disease in children.



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## CHAPTER 40

### THE MOLDLIKE BACTERIA (ORDER ACTINOMYCETALES)

WE NOW turn our attention to another of the great orders of bacteria, the Actinomycetales which, in some respects, seem to represent a slightly more advanced stage in the evolution of the plant kingdom than do the Eubacteriales, although some authors regard them as degenerate molds.<sup>1</sup> The name of this order is derived from the term *Actinomyces* used by Hartz in 1878, to describe the organism (*Actinomyces bovis*) causing "lumpy jaw" of cattle. It means "ray fungus" and is descriptive of the peculiar, radial or sunray-like arrangement of the threads of *Actinomyces* which make up the granules found in the pus from the lesions of the disease actinomycosis (see Fig. 316).

Those who regard the primitive waters of the earth as the place in which life originated, and the Eubacteriales as the most primitive of known plants, find some support for that view in the numbers of motile, aquatic and marine forms in that order. The Eubacteriales are essentially fluid-inhabiting organisms. The Actinomycetales, on the contrary, are almost entirely restricted to the soil and are nonmotile, *i. e.*, are not equipped for swimming. On the contrary, most of them are especially equipped for growth on solid surfaces exposed to the air, requiring relatively little moisture in their nutrient substrate. Only a few species are anaerobic.

In contrast with the Eubacteriales, which do not branch normally, the order Actinomycetales contains many species which normally develop branching cells, this habit being extended in the

genus *Actinomyces* to the formation of aerial hyphae (Fig. 317) strongly suggestive of molds,\* and still further resemble the latter in reproduction by conidia. The hyphae are nonseptate.

The conidia (often improperly called spores) are not as resistant as bacterial spores, being killed by ten- to thirty-minute exposures to 70° to 75° C., temperatures only slightly higher than those required to kill the vegetative mycelium. The conidia, when present, are usually formed by the division of terminal sections of aerial hyphae into short, oval or round segments singly or in chains. In many species the formation of conidia is accompanied by a corkscrew twisting of the filament so that a very curious, curled appear-

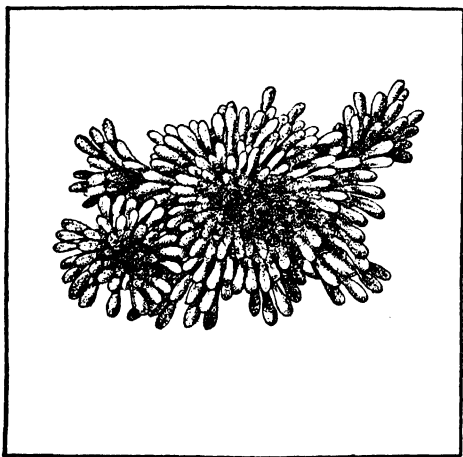


Fig. 316.—*A. bovis* from tissues, showing club-formation and radial arrangement giving rise to the name *Actinomyces*. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

ance is given to the mycelium (Fig. 317). The formation of club-shaped enlargements at the ends of filaments or cells is a very characteristic feature of many of the Actinomycetales.

A striking difference between the moldlike Actinomycetales and the true molds (Eumycetes) lies in the minuteness of the filaments and the absence of visible nuclei in the former. Granules of volutin and other substances, as well as vacuoles within the older mycelia of *Actinomyces*, are often seen and have probably been mistaken by some investigators for nuclei. In diameter, the filaments of

\* If not already familiar with the general structure of molds, the student is advised to read Chapter 9 on molds.

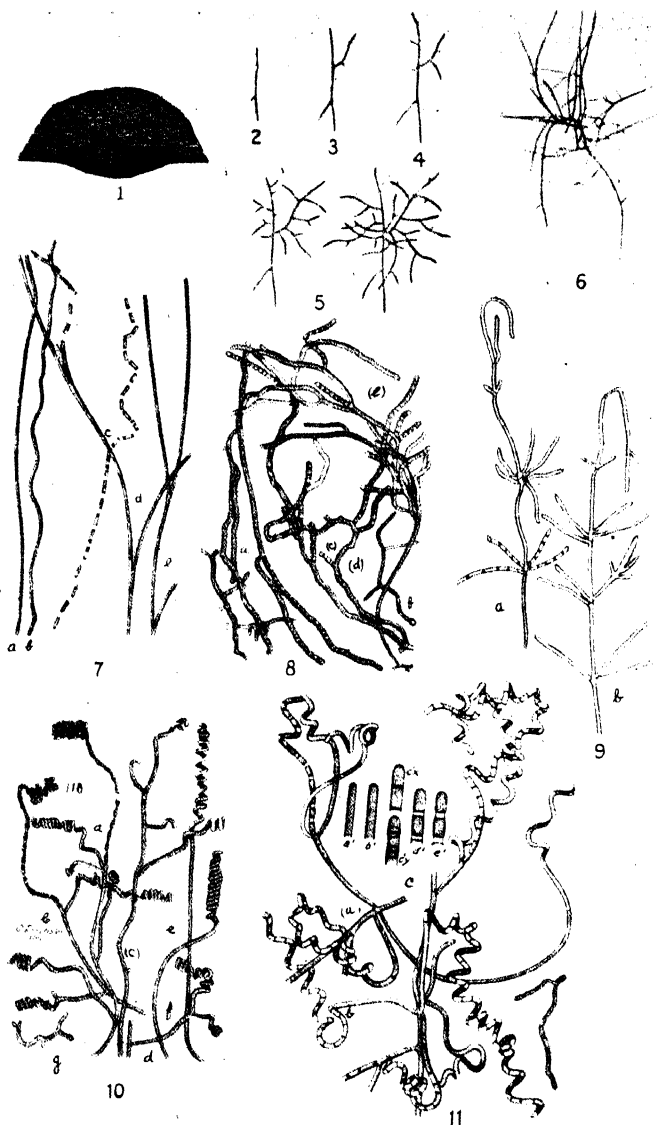


Fig. 317.—Details of *Actinomyces* growth and structure. 1, Cross section of colony; 2–6, successive stages in growth from a single spore; 7–11, various types of mature mycelium, showing different kinds of branching, conidia formation, twisting, etc. (1–6 from Lieske; 7–11 from Drechsler and Waksman.)

Actinomycetales are of the same order of magnitude as those of the true bacteria, seldom exceeding 1 or 2 microns. Their length may greatly exceed this, but does not approach that of true mold hyphae.

The Actinomycetales show their relationship to true bacteria in a tendency of nonconidial filamentous species to break up into bacillus-like fragments, referred to by Cumberland as "fragmentation bodies."<sup>2</sup> These may be of irregular shape and size—round, oval, thin rods, "clubs," and so on. Fragmentation is very characteristic of the pathogenic forms and, in these, mycelium and conidia formation are inconspicuous or absent. This is especially true when cultivated on artificial media. The saprophytic varieties, on the contrary, usually develop a distinct aerial mycelium, form abundant conidia, and fragment much less readily.

There is no differentiation of sexes, such as is seen in molds, and there is neither zygospore nor endospore formation. Actinomycetales are typically gram-positive, especially young growth. Several species are acid-fast.

**Classification of the Actinomycetales.**—Variability of practically all the properties, such as form and arrangement of mycelium and conidia, pigments, fermentations and colony form, which could be used for classifications of the organisms of this group, especially the saprophytic sorts, makes the taxonomist's task very difficult.<sup>3</sup> For this reason many different groupings have been proposed, each having its advantages and disadvantages.<sup>2, 3, 4, 5, 6, 7</sup> Synthetic media of constant and known compositions, and rather poor in nutriment, induce the appearance of differential characters and tend to stabilize them, while "rich" organic media tend to cause confusing changes. No attempt at a detailed classification will, therefore, be attempted here, but certain general segregations which depend on fairly obvious and constant properties can be used to advantage in this discussion.

Largely for convenience in discussion and study, and not because such lines of differentiation are sharply defined in nature, the order of Actinomycetales is divided at present into two families with respect to filament formation. Those which form long, often-branching, filaments are grouped in the family Actinomycetaceae. In a second family (Mycobacteriaceae) are grouped two genera in which filament formation is less conspicuous or absent, the tendency being reduced to occasional branching of cells. This branching is believed to be an abnormal process in one genus (*Corynebacterium*) and the position of such organisms in the order of normally

branching Actinomycetales is probably untenable. The family includes dangerous human and animal pathogens (genera *Mycobacterium* and *Corynebacterium*) as well as soil-inhabiting saprophytes.

#### FAMILY ACTINOMYCETACEAE

Most of the members of this family, notably the *Actinomyces*, are saprophytes, living in the soil as scavengers and attacking complex organic substances of a great variety, such as cellulose, protein, starches, fats, and even carbolic acid (phenol), naphtha-

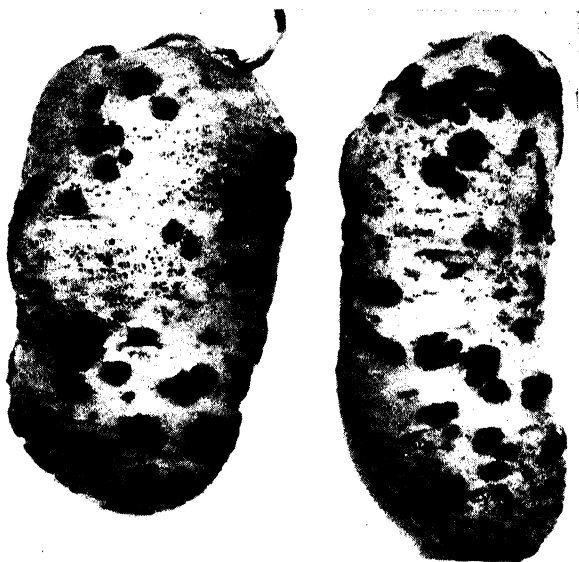


Fig. 318.—Potato tubers affected with common scab. (Photo by Bailey, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

lene, rubber and cresol as sources of energy or carbon. Their food requirements are otherwise very simple. There are four genera.

**Genus *Actinomyces*.**—This genus, as at present classified, comprises some sixty or more species. Eight are parasitic in man or animals, one (*Actinomyces bovis*) causing "lumpy jaw" (actinomycosis) of cattle, one (*Actinomyces madurae*) causing "madura foot" or mycetoma in man, others causing tuberculosis-like diseases or ulcerative lesions in animals. Some are plant parasites, "scabies" of potatoes being an example of an actinomycotic disease of plants (Fig. 318).

All are found in the soil or dust, performing important and valuable scavenger work by assisting in the decomposition of dead plant and animal remains, and in their reduction to compounds such as ammonia, hydrogen sulfide, organic acids, alcohols, amines, dextrans, glucose, etc., utilizable by other soil bacteria and by plants.<sup>8</sup> The *Actinomyces* are the most moldlike of the family Actinomycetaceae, often forming beautifully pigmented aerial hyphae, bearing conidia as described above. The conidia are whitish, or brown-gray. The growths give off musty odors such as are noticed in damp cellars and in dense woods. Their pigments, the arrangement and form of their hyphae, and their biochemical action on various substances are used to differentiate the species.

Cultivation from soil is not difficult, but slow development may permit overgrowth by other, more rapidly multiplying, bacteria if the latter are present in large numbers in the culture. Differentiation of species is often difficult. On organic media, like potato or infusion agar, differentiation of species is poor because all tend to resemble one another. However, on synthetic media they show useful and relatively constant differential characters, like pigmentation. Conn and Conn have shown that the fact of pigment production *per se*, and the chemical nature of the pigment are more reliable bases of classification than the colors, since color in a given species may depend on pH of medium and other environmental factors.<sup>7</sup> The best results with soil forms, especially in the development of colors and conidia, are obtained on media such as Czapek's,\* which, except for a source of carbon, like sucrose, contain very minute amounts of nutrient material, chiefly inorganic.

The colonies on agar are usually flat and tough, adhering strongly to the medium because of subsurface hyphae. Such colonies are usually from 2 to 5 mm. in diameter, somewhat conical, often papillate, and frequently the surface is thrown into radial folds or ridges (Fig. 319).<sup>9</sup> These colonies resemble very minute mold colonies but are more compact. They give off a distinctive, musty

\* Czapek's solution (modified by Dox and Thom):

Sucrose .....	30.0 gm.
NaNO <sub>2</sub> .....	2.0 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	1.0 gm.
MgSO <sub>4</sub> .....	0.5 gm.
KCl .....	0.5 gm.
FeSO <sub>4</sub> .....	0.01 gm.
H <sub>2</sub> O .....	1000.0 cc.

Agar may be added to 1.5 percent concentration. The reaction should be around pH 7.8.

odor. Some species form more friable, less tenacious colonies. The surface of the colonies is usually dry-looking and older colonies may be powdery, velvety or woolly, due to the aerial hyphae and conidia.

In order to preserve such material for study, Henrici recommends that agar, inoculated with spores of the desired species, be spread thinly on sterile slides and incubated in a Petri dish with sterile, water-saturated cotton.<sup>3</sup> After growth has occurred, the slides are immersed in methyl alcohol and stained. This procedure preserves the relations of all parts intact.

Growth is usually best at temperatures around 25° C., although there are some thermophilic soil species growing well at temperatures as high as 60° C. All soil species are strict aerobes. Growth is best in material of an alkaline reaction, occurring at *pH* as high as 8 or 9, and being greatly depressed by acid reactions of around *pH* 5.

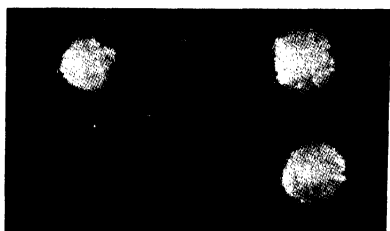


Fig. 319.—Typical colonies of *Actinomyces*. (about 2×.) (Bibby and Knighton, *Jour. of Inf. Dis.*, Vol. 69.)

It is obvious that liming of acid soils will encourage growth of the saprophytic actinomyces, resulting in increased fertility due to the activities of these organisms in decomposing complex organic materials, including cellulose, so that other bacteria and farm crops can make use of them. The various species causing “scab” of potatoes and other root crops are also encouraged by this procedure so

that one must consider the nature of the crop to be raised and soil *pH* before indiscriminate liming. As the soil actinomyces are aerobic, it is apparent why draining swamp lands increases their fertility.

**Pathogenic Actinomyces.**—A number of the *Actinomyces* are dangerous pathogens, although they live for the most part in the soil much as do the saprophytic and harmless species.<sup>10</sup> The species (*A. bovis*) causing “lumpy jaw” (actinomycosis) of cattle and occasionally similar lesions elsewhere in man (*A. hominis*), and the species (*A. madurae*) causing a somewhat similar disease of the feet (“Madura foot”) in certain tropical regions, are probably best known. Other well-known species causing related diseases in man or animals are *A. asteroides* (tuberculosis-like disease in man) and *A. farcinicus* (tuberculosis-like disease of cattle).

*Actinomyces bovis* (also *A. hominis* and *A. madurae*) is probably



introduced into the flesh by thorns, splinters and the like, contaminated with soil containing the species capable of growing in the animal body and causing the various forms of actinomycosis. Swellings are produced and the surrounding tissues become hard and indurated; hence the term "wooden tongue" often used colloquially in describing actinomycosis of the tongue in cattle. Eventually the infected tissue becomes riddled with abscess-like cavities which often coalesce and ulcerate on the surface (Fig. 320). They are filled with pus. Often the adjacent bony structures are invaded also (Fig. 321). The disease is a chronic one.



Fig. 320.—Transverse section of a part of the lesion of actinomycosis showing numerous abscesses. "Lumpy jaw."

"Madura foot" is a disease of man, common in many tropical countries.\* It is similar in many respects to "lumpy jaw" except that the lesions occur in the feet, the organism (*A. madurae*) probably being introduced through cuts or insect bites (Fig. 322). It is confined almost entirely to natives who go barefooted.

The actinomyces concerned in "lumpy jaw" and "Madura foot" tend to form in the pus large (0.5 to 5 mm. diameter) masses of growth with a central core of matted mycelia, the tips of which project at the exterior much like the spines of a sea urchin or a

\* Madura is the name of a town in India where this disease was first described.

chestnut bur. These tips, unlike a bur or spines, characteristically enlarge and become club-shaped. These masses or granules of mycelial growth, because of their yellow color, are often called "sulfur granules." Crushed between two glass slides, their mycelial



Fig. 321.—"Lumpy jaw." Lower jaw of cow showing involvement of bone, with great swelling. (M. Schlegel.)



Fig. 322.—Madura foot.

and radial structure is evident and is the basis of the name "Actinomyces" (ray-fungus) first used by Hartz in describing these organisms in 1878 (Figs. 316 and 323).

The exact relationships of the causative organisms of the various forms of actinomycosis are not entirely clear. It is not improbable

that the same or similar conditions may be caused by any one of several closely related kinds of actinomycetes.

The species mentioned above, while similar in many respects, may be differentiated by their staining reactions, relationships to oxygen, and some other properties (Table XVII).

All of these species are heterotrophic and grow best in serum infusion media, cooked meat or coagulated serum.

*Actinomyces bovis* is not strictly anaerobic. It may be cultivated by several of the methods described in the chapter on anaerobiosis



Fig. 323.—“Sulfur granule” from a case of actinomycosis. (Wright.) (Photograph by L. S. Brown.)

but growth is often slow and mycelium may be restricted and it is difficult to maintain the organisms alive. An atmosphere containing 10 percent carbon dioxide is preferable to strict anaerobiosis. It is said to be sometimes accompanied in actinomycosis lesions by an easily cultivated, aerobic, harmless species spoken of as *A. graminis*. This, however, is not certain and the supposed *A. graminis* may be aerobic strains of *A. bovis*. Upon injection of *A. bovis* cultures into cattle, the disease may sometimes be reproduced, but ordinary laboratory animals are not readily infected.

*Actinomyces madurae* is readily cultivated on infusion agar, forming creamy white, yellow or red colonies which often develop aerial mycelia and conidia.

*Actinomyces asteroides*, likewise, is readily cultivable, the growth resembling that of the saprophytic species of *Mycobacterium*. Only occasionally is aerial mycelium formed and conidia are seldom if ever produced. *A. asteroides* cultures are very pathogenic for laboratory animals. In human beings and animals with lung lesions, these organisms are seen in the sputum as acid fast filaments and as bacilli almost indistinguishable from *Mycobacterium tuberculosis*. *A. asteroides* and a number of similar species are found in the soil.<sup>10</sup>

**Genus Actinobacillus.\***—In this place we may mention the genus *Actinobacillus*. The systematic position of the genus is not

TABLE XVII  
CHARACTERS OF PATHOGENIC ACTINOMYCES

Species	Staining Reaction	Relation to Free Oxygen	Pigment
<i>Actinomyces bovis</i> . . . . .	nonacid-fast (gram +)	microaerophilic or prefers 10% CO <sub>2</sub>	cream yellow
<i>Actinomyces madurae</i> . . . . .	nonacid-fast (gram +)	aerobic	orange red
<i>Actinomyces asteroides</i> . . . . .	acid-fast	aerobic	yellow red
<i>Actinomyces farcinicus</i> . . . . .	acid-fast	aerobic	yellow white

at all clearly defined. For convenience we shall include it with our discussion of Actinomycetaceae.

The genus *Actinobacillus* includes two species, the pathological relationship of which to the *Actinomyces* and *actinomycosis* is obscure but interesting. These species, *Actinobacillus lignieresii* and *A. actinomycetem-comitans*, are small, gram-negative rods. They can grow aerobically on ordinary culture media but growth is much better in serum broth or serum gelatin under an atmosphere containing about 10 percent carbon dioxide. Although associated with actinomycosis they do not at all resemble true *Actinomyces*, and especially they do not resemble *Actinomyces bovis*.

*Actinobacillus lignieresii* causes a disease much like "lumpy jaw,"

\* Because of similarity of names, the student must guard against confusing *Actinobacillus* with any of the *Actinomyces*.

but confined to soft tissue, which is probably often confused with true actinomycosis. "Wooden tongue" of cattle is a common manifestation of the infection. The granules of bacilli found in the pus closely resemble the sulfur granules occurring in the lesions of true actinomycosis. Unlike the granules of true actinomycosis, however, these granules are seen to consist of masses of slender, nonmotile gram-negative bacilli and not of the gram-positive, radial, mycelial network of *Actinomyces bovis*. *A. lignieresii* can infect man.

*Actinobacillus actinomycetem-comitans* is often found associated in enormous numbers with *Actinomyces bovis* in the lesions of actinomycosis, but is thought to have no etiological relationship to the disease. It is not pathogenic for laboratory animals. It is found chiefly or only in human beings. In broth cultures it tends to form granules suggestive of sulfur granules.<sup>11</sup>

**Genus Erysipelothrix.**—The position of this genus in the order Actinomycetales is dubious. The important species (*Erysipelothrix rhusiopathiae*) of this genus is the cause of the destructive disease called *swine erysipelas*, which may, at times, also affect man and other animals, including fish.<sup>12</sup> The organism is nonmotile, micro-aerophilic, nonbranching, gram-positive, and nonspore-forming but resistant to drying and conditions in shaded soil. It may resist smoking and pickling processes. Sunlight kills it. It may be cultivated from the internal organs, skin, joints, urine and feces of diseased animals in meat, blood or serum media. Hutner devised a simplified medium.<sup>13</sup> The rods as first isolated from infected tissues are short (1 to 2 microns), slender and straight, or slightly curved. In artificial media long rods (4 to 15 microns) appear. A zone of green and hemolysis is seen around deep blood agar colonies, suggestive of alpha-type streptococcus colonies. The organisms are not especially versatile metabolically. A few substances are fermented with acid production, among them lactose, fructose and dextrose.<sup>14</sup>

*E. rhusiopathiae* lives in soil, and swine are readily infected by living on infected soil but not by injections of pure cultures. Characteristic lesions are "knotty" joints and patches on the skin. Passive immunization is of some value. Active immunization with a combination of living organisms and immune serum is being tried with some success but, due to the danger of spreading the infection, is restricted to certain experimental areas.

**Genus Fusobacterium.**—These are strictly anaerobic organisms, killed by even an hour of exposure to air. Their relation to the order Actinomycetales seems remote. They are nonspore-forming, gram-negative, slender, nonbranching rods with pointed tips (Fig. 324).

They are common in the normal mouth, and may be seen in smears of material scraped from around the teeth. Their study has been difficult and the genus is not fully known. They require special media, some species growing on blood agar,<sup>15</sup> others on a 10 per cent potato-extract agar containing cysteine.<sup>15a</sup> Growth requires about a week at 37° C. Several morphological, fermentative, and antigenic varieties have been differentiated.

The significance of these organisms is not known. As will be seen in the section on spirochetes (page 693), *F. plauti-vincenti* (the type species) is regularly associated with certain species of *Borrelia* in ulcerous conditions of the mouth, often called "trench mouth" or Vincent's angina, but their etiological relation is obscure. Some species of *Fusobacterium* occur in lung abscesses.



Fig. 324.—Fusiform bacteria from the mouth. ( $\times 1000$ .) (Berry and Hine, Jour. of Bact., Vol. 34.)

**Genus Proactinomyces.**—This genus contains several species, some of which were formerly included in the genus *Actinomyces* because of filament formation. Others were classed with *Mycobacterium* because they form no *extensive* filaments. They are non-motile, non-sporeforming, aerobic, non-acid-fast and gram-positive. The genus may be regarded as intermediate between the true-bacterium-like genera, *Mycobacterium* and *Corynebacterium*, and the mold-like *Actinomyces*.<sup>16</sup> At the borderlines of these divisions there is much indecision and difficulty of classification. While many resemble the *Actinomyces* in producing definite, branched mycelial hyphae, others differ in that these mycelial forms are found only in recently isolated cultures, being soon lost on artificial cultivation. Conidia are not formed—a *differential point of basic importance*. The mycelia, when formed, usually fragment very readily into bacillus-like forms and continue to grow in this manner. In

this condition the *Proactinomyces* resemble the mycobacteria. The individual cells are slender, sometimes showing swellings or a tendency to branch.

Like the *Actinomyces*, the organisms live in the soil as saprophytes and often have the peculiarity of being able to utilize various paraffins and phenol and related compounds as sources of carbon or energy or both and will often outgrow other bacteria in atmospheres containing the vapors of these substances. Some of them are facultative autotrophs.

It is likely that many soil organisms of the general type of *Actinomyces* or *Mycobacterium* might be classed with the *Proactinomyces*, but it is evident that these differentiations are extremely difficult, and that the three genera grade into one another and have no distinct boundaries.

#### FAMILY MYCOBACTERIACEAE

The second of the two families of the order Actinomycetales is called Mycobacteriaceae and comprises two important genera, *Mycobacterium* and *Corynebacterium*. The former are definitely acid-fast organisms, the latter, nonacid-fast. The organisms of both genera are gram-positive, nonmotile, generally aerobic or microaerophilic, nonspore-forming rods. Morphologically, they are much like ordinary bacteria except for being somewhat curved and spindle-shaped and showing club-shaped and beaded cells, and a few species which occasionally branch so that X, Y, L and similar forms are sometimes seen. Most of them are harmless saprophytes living in the soil and decaying organic matter or in the nose and throat of man and animals. None of them forms conidia or aerial hyphae. In these respects, the group is seen to be more closely related to the true bacteria than are the *Proactinomyces*. Recent studies<sup>2</sup> indicate that *Corynebacterium* should be classed in the Eubacteriales with the *Propionibacterium* and *Lactobacillus*.

**Genus *Mycobacterium*.**—The cells of mycobacteria are slender (0.1 to 0.3 microns), and vary in length from 2 to 5 or more microns. They are often curved slightly. Acid-fastness is possessed in varying degrees by different members of this genus and is particularly marked in the pathogenic species. A morphological feature often seen in mycobacteria stained by this method is the presence of intensely colored granules within the cells, usually of about the same diameter as the cell. Sometimes six or eight granules are seen in a single cell.

The Ziehl-Neelsen acid-fast stain is of great value in the identi-

fication of mycobacteria, although the filaments of some *Actinomyces* and *Proactinomyces* are somewhat acid-fast and, when fragmented, may closely resemble true mycobacteria.

**Nonpathogenic Species of Mycobacterium.**—Saprophytic mycobacteria may be found in properly stained smears of material from preputial secretions (*Mycobacterium smegmatis*) or from folds of the skin as in the buttocks or axillae. Saprophytic acid-fast bacilli are also found in butter (*Myco. butyricum*), in manure (*Myco. stercoris*), or on hay (*Myco. phlei*). Many species are also found in the soil where they act as scavengers and bring about organic decomposition.



Fig. 325.—Electron microscope picture of *Mycobacterium tuberculosis* ( $\times 32,500$ ). Compare with Figure 31. (Courtesy of R. C. A., Camden, N. J.)

These organisms are readily cultivated at 25° to 30° C. on infusion agar containing glycerin or on coagulated egg or serum. Yellowish or reddish pigments are common. The growth is fairly rapid as a rule (forty-eight to ninety-six hours), a character differentiating these harmless species from parasitic species such as *Myco. tuberculosis*, which grow very slowly on such media, appearing only after ten days to three or four weeks. The colonies of the saprophytic species are usually soft and moist, those of the species pathogenic for man and cattle are dry, rough, crumbly and dull in appearance (Fig. 326). There are, however, exceptions in each



group. Differentiation among the saprophytic species is difficult and depends largely on a knowledge of the source of any given strain.

The mycobacteria living in the soil have metabolic powers very similar to those of the soil *Proactinomyces*. Among their distinctive properties is ability to utilize the hydrocarbons present in petroleum,<sup>17</sup> as well as phenol and cresol. They will outgrow other soil bacteria in the presence of benzene and phenol vapors, which they seem to utilize in their metabolism as a source of energy. Some are said to have autotrophic metabolic properties, using nitrates as a source of nitrogen, and carbon dioxide of the atmosphere as a source of carbon. Some species produce pigments with vitamin A properties, synthesizing them from minerals and petroleum.<sup>17a</sup>

**Mycobacterium tuberculosis.**—This organism, causing tuberculosis, is a rod-shaped bacterium, varying somewhat in size and form but characteristically somewhat curved, slender, and tending toward a spindle shape (Fig. 325). Under some circumstances it shows a tendency to branch. When properly stained, tubercle bacilli are often seen to contain granules. They are strongly acid-fast.

Tubercle bacilli do not form spores, but may remain alive for long periods outside the human or animal body. In dried sputum in dark corners they may live six to eight months. In particles of dried and powdered sputum which can float through the air as dust, they can also remain alive for days and may be inhaled. Exposure to sunlight for a few hours kills them and so does pasteurization.

**Types of Tubercle Bacilli.**—There are several kinds of tubercle bacilli, varying according to the animal infected. For example, there is a human type (*Myco. tuberculosis*, var. *hominis*) and a bovine type (*Myco. tuberculosis*, var. *bovis*) which grow well at 37° C., a bird or avian type (*Myco. avium*) which grows well at about 40° C., and turtle (*Myco. chelonae*), fish (*Myco. marinum*) and other cold-blooded animal types which grow well at lower temperatures (18° to 30° C.).<sup>18</sup> All look alike microscopically and may be cultivated on similar media, although the human type grows well on glycerin media whereas the bovine type does not do so well. However, there

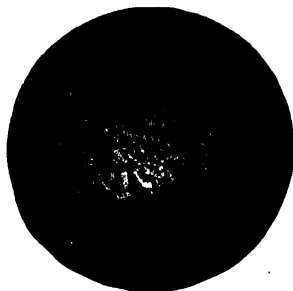


Fig. 326.—*Mycobacterium tuberculosis*, human source. Mature colony on glycerol-agar. Actual size. (Swithinbank and Newman.)

are other means of cultural and pathological differentiation. The cold-blooded animal types, for example, do not as a rule infect the warm-blooded animals or birds and vice versa. Human and bovine types seem to infect man and guinea pigs with equal ease but differ in their effect on rabbits, the bovine killing the rabbit within four to six weeks, the human usually not killing at all, or much more slowly. In glycerin broth, after four to six weeks' incubation, the human types will generally have produced an acid reaction, the bovine types not.

**Laboratory Diagnosis of Human Tuberculosis.**—As is well known, tubercle bacilli often occur in large numbers in the sputum of persons with active pulmonary tuberculosis. For diagnostic purposes the organisms may be demonstrated there or in other material such as urine, pleurisy fluid or feces, depending on the organs affected. The methods available for demonstrating the tubercle bacilli in pathological material are (a) microscopical examination of smears of the material, stained by the acid-fast method; (b) cultivation on media specially adapted to the growth of the tubercle bacillus and (c) by the inoculation of guinea pigs or rabbits, followed by observation of the development of tuberculosis in these animals.

*Cultivation of Tubercle Bacilli.*<sup>19, 20, 21</sup>—When cultivating\* from sputum or other contaminated material, it is necessary to kill or

\* **Petragnani's medium for *Mycobacterium tuberculosis* (Frobisher's modification):**

**Mixture A**

Milk . . . . .	225.0 cc.
Potato starch . . . . .	9.0 gm.
Peptone . . . . .	1.5 gm.
Diced potato . . . . .	150.0 gm.

Heat in double boiler one hour, stirring occasionally.

**Mixture B**

Eggs . . . . .	8.0
Glycerin . . . . .	18.0 cc.
2 percent aqueous malachite green . . . . .	15.0 cc.

Mix well.

**Mixture C**

Dextrose . . . . .	1.5 gm.
Asparagine . . . . .	1.5 gm.
Water . . . . .	50.0 cc.

Warm till dissolved.

Mix A, B and C in a bowl and strain through one layer of gauze. Tube and sterilize in slanting position. Sterilize in same manner as Löffler's medium. Cotton plugs should be saturated with sterile vaseline to prevent drying out.

inhibit the growth of the other bacteria present, otherwise they will overgrow and kill the tubercle bacilli. The material, especially sputum, is therefore treated with an equal volume of 5 percent sodium hydroxide for thirty minutes at 37° C. This kills nearly everything but tubercle bacilli. The fluid is then neutralized with sulfuric acid and centrifuged, the sediment being transferred to slants of suitable medium. In order to inhibit the growth of any bacteria resisting the sodium hydroxide, certain dyes are added to the medium. Brilliant green, crystal violet, congo red, etc., will prevent the growth of many bacteria but not of tubercle bacilli.

*Inoculation of Animals with Tuberculous Material.*—If desired, the sediment from sputum or other material, prepared as described in the preceding paragraph, may be injected into guinea pigs. The development of tubercles in the animals is a slower but probably more sensitive means of demonstrating tubercle bacilli than microscopic search or cultural methods. If even a very few tubercle bacilli are present they will infect the lymph glands in the neighborhood of the injection and in three to six weeks the glands will be found enlarged and caseated (cheesy gangrene) and full of acid-fast bacilli. The spleen may also be invaded.

**Tuberculosis.**—The tubercle bacillus is responsible, annually, for more deaths in the United States than any other known micro-organism. For example, during the year ending March 31, 1944, among every 100,000 industrial policyholders of the Metropolitan Life Insurance Company, there were reported 44 deaths from tuberculosis, as compared with 0.6 for scarlet fever, 0.7 for whooping cough, 0.2 for typhoid fever, and so on, and this figure (44) is much lower than prevailed about forty years ago when over 200 deaths from tuberculosis in every 100,000 persons were reported annually in New York State alone. There are, in addition, large numbers of persons who become infected but who do not die of tuberculosis, and many cases which are never reported.

*Tubercles and Tuberculosis.*—When tubercle bacilli gain a foothold in a susceptible animal or person, whether by inhalation or by way of the alimentary tract, the tissues where the bacilli localize immediately begin to react against the organisms in a very characteristic way. Numbers of the tissue cells begin to grow around the bacilli in an attempt to incarcerate them or wall them off. This tiny, pearly gray mass of cells with tubercle bacilli at the center is called a *tubercle*. If the resistance of the host is low, the tissues are unable to arrest the bacilli and they continue to grow, killing the surrounding cells. Numbers of adjacent tubercles may coalesce.

The dead tissue at the center of such masses of tubercles becomes cheesy and yellowish and is said to be *caseated* (Fig. 327). If the tubercle is in a lung, the necrosis (death of tissue) may extend till it invades and breaks through the wall of a bronchus, and then the



Fig. 327.—Pulmonary tuberculosis. The lung has been cut through the center and we are looking at a section. The section shows masses of caseated tubercles. The cavities were formed by the softening of such masses and the material was cast off as sputum. (MacCallum.)

caseous material, which may contain millions of living tubercle bacilli, is discharged with the sputum by coughing (Figs. 327 and 31).

Often the caseous process breaks through the wall of an artery and then hemorrhage of the lung occurs, which may be fatal. In

human beings these tuberculous processes tend to heal and to form life-long scars, whether the patient recovers or not.

*Bovine Tubercle Bacilli and Milk.*—When cattle become infected with the bovine variety of tubercle bacilli, the microorganisms are often widely disseminated through the animal and frequently localize in the udder. Milk from infected cattle often contains enormous numbers of tubercle bacilli and if drunk by susceptible people or calves will cause tuberculosis of the abdominal organs and eventually of the lungs, and sometimes tuberculous meningitis. Human infection with bovine tubercle bacilli is found especially in countries where veterinary inspection of dairy herds for the control of tuberculous cattle is not as effective as it is in this country. Farmers' organizations all over this country are constantly cooperating with the United States Government in an effective effort to stamp out tuberculosis of cattle. Pasteurization kills bovine and human tubercle bacilli. There is now very little bovine tuberculosis in this country.

*Distribution of Human Tubercle Bacilli.*—The human tubercle bacillus is so widely distributed among civilized men that nearly everyone becomes infected with it early in life, especially dwellers in large cities. Infection of infants probably results from contact with infected parents or other members of the household who handle the baby. Kissing, the use of improperly washed eating utensils, sleeping with infected persons and the inhalation of infected dust all may result in the transmission of human tubercle bacilli, not to mention the transmission effected by saliva droplets during coughing and the distribution of infectious saliva by the hands.

A very large percentage of persons are able to resist extensive invasion under conditions of good nutrition, rest, and shelter, however, and the only evidence of infection is a small scar in the lung which is carried through life.<sup>23</sup> This has been proved by examination of the lungs in thousands of autopsies. It seems that only those whose resistance is low, due to malnutrition, chronic disease, overwork, and other causes, actually develop clinical tuberculosis. Pearl has adduced evidence that physical characteristics predisposing to tuberculosis may be inherited.<sup>22</sup> War, with its devastation, overwork and malnutrition is always followed by a great increase in tuberculosis, and the increase in tuberculosis in occupied Europe in 1944 was a scandal in the public press.<sup>24</sup>

**Tuberculin Reaction.**—In a previous chapter it was pointed out that the presence of foreign proteins, including bacterial protein,

in the blood or tissues of the body often induces, in addition to antibodies, a hyper-reactive or hypersensitive condition known as an *allergic state* toward that specific protein. It was also stated that by injecting or scratching a small amount of the protein in question into the skin of a person allergic to that protein, a peculiar, local, inflammatory reaction will be elicited, showing the patient's allergic condition.

This local reaction is very readily demonstrated in persons who have had tubercles in their tissues, whether healed or not. As has been stated, nearly every person, by the time he reaches adult life, has had some tuberculous infection. Sensitivity to the protein of the tubercle bacilli may therefore be demonstrated in a large proportion of adults by scratching into the skin tuberculo-protein in the form of dead tubercle bacilli, or the *sterile filtrate of broth in which they have grown*, or purified protein derivatives (P. P. D.) extracted from the bacilli directly. These tuberculo-proteins are called *tuberculin*. A red, itching spot appears in twenty-four to forty-eight hours, and the reaction is called a *tuberculin reaction*.<sup>25</sup>

The tuberculin reaction is of limited value in the diagnosis of tuberculosis in adults because many give a positive reaction, indicating a previous infection which has usually long since healed. Some persons may lose this reactivity unless reinfected. Young children, however, who give a positive tuberculin reaction are likely still to have an active tuberculous process.<sup>26</sup> The reaction was first described by von Pirquet and is sometimes spoken of as the von Pirquet reaction.

A method of injecting the proteins *into* the skin, similar to that used in the Schick and Dick tests, was devised by Mantoux and bears his name. It is preferable to the von Pirquet method since it is more accurate, and it is widely used in studies of the development and transmission of tuberculosis.

The student should carefully distinguish between the Schick and Dick tests, which test for *susceptibility* to *toxin*, usually before infection sets in, and the tuberculin test, which is used to detect *allergy* to an *infection already present* or healed. Skin tests for allergy, similar in principle to the tuberculin test, are used in various other bacterial infections; *e. g.*, tularemia and brucellosis.

**Leprosy.**—Although of little importance in the United States or in most of the so-called civilized countries, this disease is of great interest from several standpoints, (a) historically, (b) because of the mystery surrounding its mode of transmission, and (c) doubt concerning its true etiology.<sup>18</sup>

The disease is, of course, well known to students of the Bible. The fear and horror of lepers has been a human tradition since antiquity. Lepers were excluded from all public contacts and left to die of exposure and starvation by many peoples. They still are excluded and shunned, but provisions for their comfort and well-being are usually adequate. Much of the disease which in the dark ages caused unfortunate people to be regarded as entirely outside the pale of human consideration, and even to be put to death, was probably not leprosy at all but other disfiguring diseases such as scabies, syphilis, and eczema. One of Conan Doyle's most in-



Fig. 328.—Tubercular leprosy, showing advanced lesions of face and hands (Kuala Lumpur). (MacCallum.)

triguing Sherlock Holmes episodes depicts the tragic segregation of a noble young man because of a suspicion of leprosy. Holmes, with the aid of Watson, dispels the ugly cloud by his usual astute, deductive reasoning, and the victim is found to have nothing but a harmless skin condition called ichthyosis.

The horror of leprosy arises from the disfigurement of the body and the destruction of tissue, with scar formation, which accompanies it. (Fig. 328.) There are two forms, nervous and nodular. They may occur together. In the nodular form, large nodules develop on the surface of the body, although the infection is general. In the neural form, the nerves are affected and members be-

come devoid of sensation. Extensive injuries, as by burning, secondary infection, crushing, etc., cause no pain and patients often acquire revolting disfigurements or die from these injuries because their normal defensive reflexes do not function. Early stages of the disease include large red patches which become scaly and bronze color—not white. Hair drops out of affected areas. There is intense itching. Bones and muscles atrophy with great distortions. The face becomes horribly disfigured.

*Etiology.*—In 1874 Hansen described an acid-fast bacillus, morphologically indistinguishable from *M. tuberculosis*, obtained from lesions of lepers. It is called *Mycobacterium leprae*. The organism has never been successfully cultivated, although many claim to have done so. It is always present in leprous tissue, and never occurs in normal tissue. This is the only one of Koch's postulates on which a statement of the etiology of leprosy can be based at present. Even inoculation of leprous tissue into human volunteers, chimpanzees, etc., has never produced an authentic case of leprosy.

*Transmission.*—No one knows with certainty how leprosy is transmitted, or the incubation period. It persists in peoples who live in dirty and insanitary circumstances and who are poorly nourished and overworked. Tuberculosis is a common cause of death in lepers. Syphilis also *seems* frequent in lepers, although this may be due to the fact that leprosy causes a positive Kahn and Wassermann test. Climate seems to have no bearing on the existence of leprosy, nor does race. Children of lepers may remain uninfected if separated early enough. Contact, in ordinary business life, of lepers and non-lepers does not appear to increase the number of cases markedly, at least in areas of good sanitation and nutrition in the United States. Attendants in leprosaria which are conducted on sanitary and intelligent lines do not frequently contract leprosy. There is some evidence that certain soils harbor the infection, for example, certain parts of the Philippines, the Orient, etc., but this is certainly open to argument. Isolation of lepers is probably of no value to the population, but is a good political move and, far more important, results in good nutrition, sanitation, diet, rest, and regular and industrious habits for the lepers, many of whom perform much useful work, live long, and raise families.

*Genus Corynebacterium.*—This genus contains *Corynebacterium diphtheriae* and a few organisms pathogenic for animals such as *C. pyogenes* which is common in purulent lesions of cattle, swine and sheep, *C. equi* causing pneumonia in foals, and *C. renale* which causes a necrotic disease of the urinary tract in cattle.<sup>12</sup> A large



number of nonpathogenic species are known which are commonly spoken of as "diphtheroids" and the classification of which is not very satisfactory, due to incomplete knowledge. Many of the so-called diphtheroids may be *Propionibacter*, *Microbacterium*, or *Proactinomyces*. The name "diphtheroid" is derived from the morphological resemblance of the nonpathogenic corynebacteria to certain forms of *C. diphtheriae*.

The nonpathogenic corynebacteria seem to be of relatively little importance from any standpoint except that of causing confusion to medical and veterinary bacteriologists by appearing as contaminants and being mistaken, sometimes, for the pathogenic species. This is particularly true of *C. pseudodiphthericum* (also called

TABLE XVIII

USUAL FERMENTATION REACTIONS OF SOME OF THE COMMON CORYNEBACTERIA

Species	Lactose	Dextrose	Saccharose	Maltose	Gelatin and Milk*
<i>Corynebacterium diphtheriae</i> .....	—	+	—	+	—
<i>Corynebacterium pseudodiphthericum</i> ....	—	—	—	—	—
<i>Corynebacterium xerose</i> .....	±	+	+	+	—
<i>Corynebacterium acnes</i> .....	±	+	—	—	—
<i>Corynebacterium pyogenes</i> .....	+	+	+	+	+

\* Proteolysis.

"Hoffmann's bacillus" or *C. hoffmannii*) which occurs very frequently in the normal nose and throat. However, this organism often acts as a secondary invader causing serious complications in hemorrhagic septicemia of swine. Differentiations between some of these various species are made on the basis of fermentation reaction, pigment formation and morphology (Table XVIII).

In general, the corynebacteria are gram-positive, nonacid-fast, nonmotile, nonspore-forming, nonencapsulated and nonfilamentous. Occasionally slightly branching forms and club-shaped cells are seen especially in certain strains of *C. diphtheriae*, and it is largely for this reason that they are included in the order Actinomycetales. However, branching, which is rare, is probably due to involution and the genus should be transferred to the order of

Eubacteriales. Nearly all are aerobic, although a few species, such as *C. acnes* which has been thoroughly studied by Ketron and Burkey,<sup>26a</sup> are microaerophilic. The corynebacteria are cultivated with ease on most of the ordinary infusion media, or on coagulated serum (Löffler's medium) or blood agar, and form colonies much like those of true bacteria, although the colonies of some species are hard and brittle like those of certain of the saprophytic *Neisseria*. The saprophytic and nonpathogenic corynebacteria are widely distributed in soil, milk, dust, water, in the air, on the skin, in the wax of the ear, in the eye, nose and throat, and it is small wonder that they are frequently encountered as contaminants. Many of them form brilliant pigments like the Actinomycetaceae.

*Morphological Differentiation of Corynebacteria.*—Morphologically, pleomorphism is the rule among corynebacteria but, with the possible exception of two species found only in the intestines of roaches, it is most highly developed in *C. diphtheriae*, and in one species often called *C. xerose*, commonly found in the human eye, nose and throat.

Most of the diphtheroids may be described as rather short, plump rods of a relatively *uniform length* and with a tendency to tapered ends. Metachromatic granules (volutin) occur but are not frequent, and club-shaped cells are not a prominent feature although often seen. When the organisms are stained with solutions of methylene blue or certain other dyes, the granules, when present often appear of a color different from the body of the rod and therefore are said to be *metachromatic* (Albert's stain, Ponder's stain). These two characters (granules and club-shaped cells) differ greatly among species and in different cultures of any given species. However, the general picture of diphtheroids is one of comparative uniformity in size and staining.

*Parallelism.*—The arrangement of the cells of diphtheroids is especially characteristic. When the cells divide, the cell membrane gives way on one side. A snapping movement then occurs and the two daughter cells swing around so as to lie side by side, like the two halves of a broken, green twig which are still held together by a shred of bark. This process, continued through several divisions, often results in numbers of cells lying side by side in what is called a "palisade arrangement" (Fig. 329).

*Corynebacterium diphtheriae* differs from diphtheroids in that the cells are usually more slender, *vary greatly in length*, show much larger and more numerous and definite metachromatic granules, and more club-shaped, spindle-shaped and other irregular forms.

They often appear vacuolated or segmented, a feature commonly referred to as "barring." Palisade arrangement is much less definite in *C. diphtheriae* than in diphtheroids, irregular groupings suggestive of the characters used in Chinese printing being an especially useful diagnostic feature in addition to those mentioned above. Indeed, the morphological features of *C. diphtheriae* are so striking that it is often the practice to confirm diagnoses of diphtheria on this basis alone.

**Diphtheria.**<sup>27</sup>—Unlike scarlet fever, pneumonia and pertussis, all of which have more or less multiple etiologies, diphtheria is a very specific disease due to only one species of organisms, *Corynebacte-*

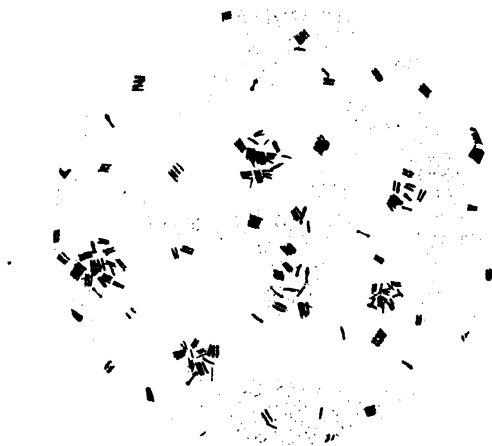


Fig. 329.—Typical diphtheroid (*Corynebacterium pseudodiphthericum*) ( $\times 900$ ). Note regularity of length and arrangement, with only an occasional beaded and club-shaped cell. (Löffler's methylene blue stain.)

*rium diphtheriae*. The organisms are transmitted in the same manner as others causing respiratory diseases. Healthy carriers are common and are doubtless sources of cases. The bacteria establish themselves on the mucous membrane of the throat and nose of susceptible persons and proceed to excrete their exotoxin.

**Toxin Production by *Corynebacterium diphtheriae*.**—*C. diphtheriae* secretes into its environment one of the most powerful biological poisons known. When the organism grows on the tonsils this poison is absorbed by the blood and may cause death unless antibodies against the toxin are already present or are developed rapidly by the cells of the patient, or are injected into him from some outside

source. This toxin is present in broth cultures of *C. diphtheriae* and can be obtained free from the bacilli by filtration. It is easily destroyed by heat, sunlight and oxygen and must therefore be kept in a dark icebox in sealed vessels. It is very poisonous to guinea pigs and, in experimental work, to chicks and other birds, as well as to human beings when injected or absorbed from infected tissues.

*Corynebacterium diphtheriae* is a classical illustration of the fact that virulence of a pathogenic organism may consist of either or

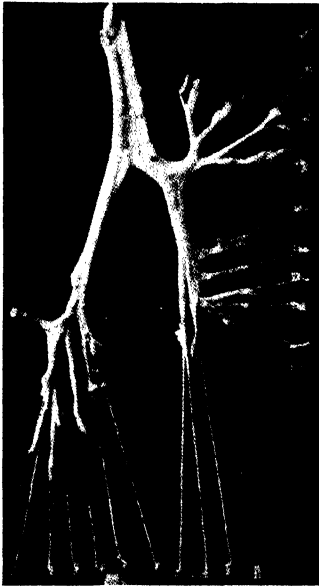


Fig. 330.—Diphtheritic membrane removed after death showing extension into air passages of both lungs. (Tull, Jour Amer Med. Assoc., Vol. 104.)

both of two factors, *aggressiveness* and *toxigenicity*. This organism, although often growing in the throat has little ability to invade the tissues beyond the mucous membrane. It is not, therefore, very aggressive. It produces death, however, because of its extreme toxigenicity. The opposite extreme is seen in *B. anthracis*.

Diphtheria is characterized by an onset with sore throat and acute illness, fever and leukocytosis (increase in number of leukocytes in the blood). The whole throat is inflamed, and white or yellowish spots or *flakes* begin to form at different points, usually on the tonsils. Many other throat infections appear thus, but in severe diphtheria the white patches may extend, often very rapidly, till the entire throat and all the linings of the air passages throughout the lungs are covered with the white material which is found to be a tough, membrane-like layer called a pseudomembrane, adherent to the mucosal surface (Fig. 330). Sometimes the inflammation produces such great swelling along with the membrane that the respiratory tract may be occluded and death from asphyxia ensue. Sometimes, in order to avoid this stoppage, a tube is inserted in the trachea below the swollen and occluded point so that air may be obtained (intubation). The diphtheritic membrane is formed under the toxic influence of the bacilli. They kill the superficial cells and cause the exudation

of a plasma-like fluid which clots and covers the mucous surface with a tough network of fibrin which enmeshes bacilli and dead cells.

**Immunity in Diphtheria.**—Children between the ages of about six months and six years are most susceptible to diphtheria, although older children and occasionally adults also have the disease. Most persons above this age develop a natural, *active* immunity probably through *subclinical* or unrecognized, mild attacks, and retain their immunity probably as a result of repeated reinfection. Their blood often contains a small amount of antitoxin and this is sufficient to combat ordinary infection.

*The Schick Test.*—It is possible to determine whether or not a person's blood contains sufficient antitoxin to protect him. The principle of the test is exactly similar to that of the Dick test for scarlet fever susceptibility. The test is made by determining ability to withstand a small dose of toxin. Of the amount of diphtheria toxin which takes four days to kill a small guinea pig (1 minimal lethal dose, as it is called), one-fiftieth is injected *into* the skin of the person whose immunity is to be tested. If the person's blood contains a sufficient amount of diphtheria antitoxin to protect him (about 0.01 unit \* per cubic centimeter of serum), nothing happens. If his blood contains too little antitoxin, a small red spot appears at the site of the injection and remains for some days. This is called a positive *Schick test*, the test having been devised by the Austrian physician, Schick. It is perfectly harmless (Fig. 331) and may do some good by stimulating antibody production.

*Active Immunization to Diphtheria.*—Children giving a positive Schick test may contract diphtheria. They should be artificially, actively immunized sufficiently so that they give a negative Schick test. Any physician or health department will do this on request. The process requires only a single injection of a specially treated diphtheria toxin. The toxin is treated with formaldehyde and alum so that it is no longer a dangerous poison but is changed to toxoid ("alum precipitated toxoid"). However, it still stimulates the production of antitoxin in the blood of the injected child (see page 311). In three to six weeks after the injection the child will usually give a negative Schick test and may be regarded as safe from diphtheria.<sup>28</sup> Inasmuch as this immunity wears away in time, it may be advisable to reimmunize in a few years. This is probably sufficient for life. Certain persons seem to remain susceptible in

\* A unit, roughly, is sufficient antitoxin to neutralize a little over 100 minimal ethal doses of toxin.

spite of apparently adequate immunization, and may contract the disease. It may be that an antibacterial as well as antitoxic immunity might help such persons.<sup>29</sup>

Adults not infrequently give positive Schick tests, but seldom have diphtheria, from which it may be inferred that such persons have the ability to respond to infection very rapidly by producing antitoxin in their blood. This power is probably due to a previous slight infection or *primary stimulus* which has conditioned their tissues to make this rapid response to a second stimulus.<sup>30, 31</sup> It



Fig. 331.—Dr. Schick, Austrian scientist, who originated the Schick test, immunizing a child at one of the special diphtheria-prevention stations established in New York. (Underwood and Underwood.)

illustrates a fundamental principle in immunity. (See section on immunology, page 312.)

*Passive Immunization to Diphtheria.*—As pointed out in the section on immunity, a person ill with diphtheria, or a child exposed to the disease by living in the same house with such a patient, may have immediate need of antibodies to combat the disease or ward off infection. Artificial, active immunization with toxoid, as described above, is too slow. Three to six weeks is too long a time to wait for protection. Endangered persons may, under special cir-

cumstances to be judged by the physician, receive immediate protection through injections of serum which contains large quantities of antitoxin. This is passive immunity and is quite transitory. After the passive immunity has worn off (one to two weeks) or even while it lasts, *active* immunization should be used as described above.

It is as though a new town, having had no time to organize and train a fire department, has a bad fire. It needs immediate protection and calls in the department of a neighboring city. After the emergency, it can build up its own department slowly as circumstances permit. The emergency protection is transitory.

**Laboratory Methods for the Study of Diphtheria Carriers and Cases.**<sup>21</sup>—The detection of diphtheria bacilli in the throat or nose of diphtheria patients, convalescents and normal persons is obviously of the utmost importance in the diagnosis and control of the disease. For this reason, special means have been devised for finding and isolating the organisms. One of the simplest cultural procedures consists in rubbing a wooden applicator tipped with cotton over the infected or suspected tonsils and nasal mucosa, and then rubbing the swab over the surface of sterile, coagulated serum (Löffler's medium\*) or sterile coagulated eggs (Pai's medium†). After twenty-four hours' incubation at 37° C. a methylene-blue-stained smear is made from the mixed growth on the surface of the medium and organisms having the characteristic morphology of *Corynebacterium diphtheriae* are sought among the myriads of other organisms present. Considerable experience is necessary for this work (Figs. 329 and 332) for it is in such material that one often encounters diphtheroids.

There is another kind of diphtheria-like organism which may cause considerable confusion. It is harmless for guinea pigs but is morphologically and culturally *indistinguishable* from *C. diphtheriae*. This is spoken of as *avirulent C. diphtheriae*.

Virulence of a corynebacterium cannot, therefore, be inferred from its typical *Corynebacterium diphtheriae*-like morphology or cultural characters. To quarantine a person merely because that person has an organism growing in the throat which is morphologically and culturally indistinguishable from *C. diphtheriae* may obviously be wrong. The virulence or avirulence of the organism

\* Löffler's medium: To 3 parts of serum add 1 part of 1% dextrose broth. Tube and sterilize 1 hour in a slanting position without releasing steam or air pressure.

† Pai's medium: For the serum in the above, substitute whole hens' eggs, and for the broth substitute distilled water. Sterilize in the same manner.

should be determined. This can be done by one of several methods. All involve the injection of the bacteria into guinea pigs or rabbits or ten-day-old chicks after isolation in pure culture.

*Tellurite Media in the Isolation of Corynebacterium diphtheriae.*—The isolation of corynebacteria from such highly contaminated material as cultures of the secretions of the throat or nose is greatly facilitated by the use of blood agar containing *potassium tellurite* and the amino acid *cystine*.\* Cystine, in small quantities, greatly favors the growth of corynebacteria. Potassium tellurite, in con-



Fig. 332.—*Corynebacterium diphtheriae*, cultivated on Löffler's medium after isolation in pure culture. Stained with Löffler's methylene blue ( $\times 900$ ).

centrations of about 0.04 percent, not only retards or completely inhibits the growth of many other bacteria but, due to little-known chemical reactions, the colonies of corynebacteria on tellurite media are characteristically black or dark gray with various concentric rings of a lighter color, depending on the species.

\* **Cystine-tellurite medium:** (McGuigan and Frobisher)<sup>32</sup>

To 1000 cc. of melted infusion agar cooled to 45° C. add	
Sheep or rabbit blood (defibrinated or citrated).....	50 cc.
Cystine.....	25-50 mg.
0.3 percent solution potassium tellurite.....	150 cc.

Mix well and pour into Petri dishes in amounts of 12-15 cc.

Since the cystine does not dissolve readily, the flask should be agitated frequently while pouring the plates.

The percentage of tellurite given has produced good results, but since not all samples of this salt are exactly alike, the strength of the solution might require some modification according to condition. Potassium tellurite solution may be sterilized by autoclaving or filtration. *Do not heat tellurite and cystine together!*



The colonies of *Corynebacterium diphtheriae* on tellurite-cystine medium are usually opaque, gun-metal in color, domed, shining and circular and about 1 mm. in diameter (Fig. 334). Sometimes they are smaller and flatter and partly translucent. While the colonies may usually be recognized by one familiar with them, the morphology of *C. diphtheriae* growing on media containing tellurite is often very atypical so that, for final identification, as well as for virulence tests, the organisms must be transferred to Löffler's or Pai's medium.



Fig. 333.—Rabbit showing reactions of “virulent” cultures with controls. The dorsal skin of the rabbit was marked in squares with an “indelible” pencil. The test injections (0.2 cc. of culture) were made in alternate squares. Five hours later the rabbit received 1000 units of antitoxin intravenously and the same cultures were reinjected in the unused alternate squares. This represented a specific control. Visible necrosis (large dark areas) developed in seventy-two hours at the sites of the first injections; very small, non-necrotic reactions appear at the sites of the control injections. (Fraser and Weld, Trans. Roy. Soc. Canada, Sec. V, Vol. 20.)

Once a pure culture has been obtained further studies of the organism are relatively simple. An important procedure is the virulence test. This distinguishes true, pathogenic *C. diphtheriae* from avirulent *C. diphtheriae* and from diphtheroids. The suspected organisms are cultivated in broth, or are grown on Löffler's medium and then suspended in 3 or 4 cc. of normal salt solution. One-tenth cc. of the culture is injected *into* (not *under*) the shaved skin of a guinea pig or rabbit. The resistance of guinea pigs or rabbits to ordinary throat bacteria and avirulent diphtheria bacilli when

injected in this manner is such that the infection so produced, if any, is seldom severe, is localized and soon heals.

If, however, *virulent* diphtheria bacilli are injected, a different and quite distinctive type of lesion is formed. In thirty-six hours the diphtheria bacilli cause the *superficial* layers of the skin to become necrotic (dead) and so loosened that they will slip off when rubbed between the thumb and forefinger. The necrosis is usually obvious to the eye. This superficial necrosis is quite widespread (1 to 3 cm.) and is not confined merely to the point at which the



Fig. 334.—Colonies of *Corynebacterium diphtheriae* on cystine-tellurite-blood agar. Plate was inoculated with a mixed culture of Löffler's medium, made directly from the throat of a patient and containing many different sorts of bacteria. This is a good example of selective bacteriostasis.

needle entered as is true of the occasional tiny abscess due to non-toxigenic bacteria. There may, on the contrary, be little inflammation, swelling, or suppuration. Eventually the necrosis extends and deepens and after forty-eight or seventy-two hours a definite ulcer is formed, covered with a crust.

As a further check upon the truly diphtheritic nature of this reaction, it must be shown that it will not occur in animals passively immunized with diphtheria antitoxin. Much experience is needed in interpreting virulence tests.

Virulence tests with impure cultures are to be avoided as misleading.

As shown by Löffler and confirmed by the author, chicks are highly susceptible to diphtheria and may conveniently be used to test virulence of diphtheria bacilli. The experiments are made with 2 cc. of forty-eight-hour broth culture injected subcutaneously.<sup>33</sup>

**Gravis and Mitis Types of *Corynebacterium diphtheriae*.**—In recent years attention has been called by Anderson, Happold, McLeod and Thompson,<sup>34</sup> British bacteriologists, to the existence of a variety of *C. diphtheriae* found by them in the city of Leeds which they state produces a much more severe and dangerous form of diphtheria than ordinary varieties. They gave the name of *C. diphtheriae gravis* to the organisms which they described. These organisms are characterized, according to them, by a peculiar diphtheroid-like morphology, an ability to ferment starch and glycogen, inability to hemolyze human erythrocytes, tendency to rapid alkalization of broth, and the appearance, on the surface of their agar colonies, of radial and concentric ridges forming a pattern suggestive of the flower of a daisy. They suggest that many cases of diphtheria which seem refractory to the effect of antitoxin are due to the gravis type of organism.

A second type of less harmful and frequently avirulent diphtheria organisms are grouped by them under the title *C. diphtheriae mitis*. These have properties generally opposite to those listed as distinctive of the gravis type. Intermediate forms possessing some of the characters of both types are also recognized.

Other authorities, as Parish, Whatley and O'Brien,<sup>35</sup> have shown that the relationships pointed out by Anderson and his co-workers are local phenomena and that, while starch-fermenting varieties of *C. diphtheriae* are not uncommon, they may or may not be of an especially virulent nature. Similar types of *C. diphtheriae* are found in the United States. The toxin which they produce has been shown by Povitsky, Eisner and Jackson<sup>36</sup> and others to be identical with ordinary diphtheria toxin. Many avirulent (harmless) gravis strains have been isolated by the author from carriers in the United States.<sup>37</sup> It seems not unlikely that hemolytic streptococci may be involved with diphtheria bacilli of any type in malignant diphtheria.<sup>38, 39</sup>

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## THE SPIRAL, FLEXIBLE BACTERIA (ORDER SPIROCHAETALES)

ALTHOUGH THE organisms to be discussed in this chapter are included as the order Spirochaetales of the class of fission fungi (Schizomycetes), it will be seen presently that they have relatively little in common with fungi or with most of the bacteria already discussed.<sup>1</sup>

**General Characters of the Spirochaetales.**—There are six (or seven) genera in the order Spirochaetales,<sup>2</sup> the members of which

vary greatly in size, structure and physiological properties but all of which possess certain features which entitle them to a position in the order. They are cylindrical in form. None forms spores, conidia, branches or filaments, and they exist as single cells. They possess several properties which relate them more or less closely with the protozoa, or unicellular animals. Only a few species have been cultivated on artificial media. All are (a) distinctly *spiral* or *wavy* in form (Fig. 335), (b) *highly flexible* and (c) *contractile*. Motility in most Spirochaetales has been thought to be accomplished without flagella, but Mudd and his co-workers<sup>3</sup> demonstrated what seem to be flagella on certain treponemas by means of the electron microscope (Fig. 336). Flagella have not been seen on other spirochetes. These characters differentiate them sharply from the order Eubacteri-

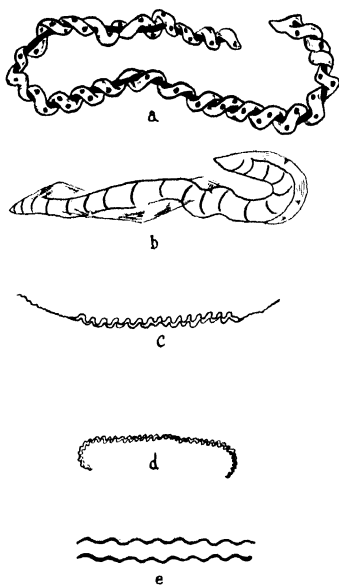


Fig. 335.—Diagram of various types of spirochete. a, *Spirochaeta*; b, *Cristispira*; c, *Treponema*; d, *Leptospira*; e, *Borrelia*. (Redrawn from Noguchi.)

ales and from the tribe Spirilleae of that order. The Spirilleae, as previously noted, consist of short, *rigid*, *flagellate* cells which are merely curved (genus *Vibrio*), or at most consist only of a few spirals (genus *Spirillum*).

The spirochetes (by which common name the members of the order Spirochaetales are collectively known), while in general not much thicker than bacteria, are usually much longer, sometimes several hundred times as long. They are, as a rule, not readily stained by ordinary dyes like methylene blue, although certain species are exceptions. When treated by Gram's method, those which respond are gram-negative. Special stains such as Giemsa's, which color protozoa, also stain the spirochetes. The larger species are conveniently observed in unstained preparations, and the



Fig. 336.—*T. pallidum* (Nichols-Hough strain).  $\times 14,000$ . Intertwined spirochetal cells. Granules, 40 to 90  $\mu\mu$  in diameter, are clearly shown within the protoplasm. One tuft of four flagella is clearly and other tufts are somewhat vaguely seen. (Mudd, Polevitzky and Anderson, Jour. of Bact., Vol. 46.)

method of negative staining sometimes gives satisfactory preparations. The darkfield apparatus is most convenient for observation of the pathogenic species when fluid suspensions are available. For tissues special silver-impregnation methods are used for staining. These will be described later.

*Motility of Spirochetes.*—Most spirochetes are thought to possess neither cilia nor flagella. Their motion is produced by a distinctly *contractile* or *streaming protoplasm* which is absent from all true bacteria. In some species of spirochetes this results in creeping movements; in others, violent *lashing* movements; in still others,

rotations. These various activities result in motions of the cells either forward or backward, a characteristic called bipolarity of motility. The possible flagella of treponemas have been mentioned.

*Structure of Spirochetes.*—The cell structure of most spirochetes is different from that of true bacteria, although certain parts are more readily demonstrable in some species than in others. Granules, thought to be associated with reproduction, have been seen with the electron microscope (Fig. 336). There is a springlike *axial filament* (said to be absent from *Saprosira* and *Cristispira*) about which is coiled the contractile protoplasm and upon which the latter acts in producing motion. Surrounding the cell is a definite membrane or periplast (said to be absent from *Spirochaeta* and doubtful in *Leptospira*). In *Saprosira* and *Cristispira* the cell is divided into *chambers* by definite, transverse, protoplasmic *septa*.

*Filterability of Spirochetes.*—Certain of the spirochetes (e.g., *Leptospira* and *Borrelia*) are readily filtrable through porcelain or kieselguhr filters and this, of course, distinguishes them from true bacteria. It is thought by some authorities that the filtrability of these organisms is due to the formation, through disintegration or otherwise, of minute granules. Possibly these are the reproductive granules referred to above. Material which has been filtered and found, on microscopic examination, to contain no visible spirochetes, has later been shown to contain spirochetes by cultural methods.<sup>15</sup> However, microscopic examination of a drop or two of filtered fluid might easily fail to detect the presence of a few spirochetes in the greater, unexamined portion.

*Bacterium-like Characters of Spirochetes.*—Characters possessed by the spirochetes in common with the true bacteria, and rare in protozoa are: multiplication by transverse fission, cultivability of some species in artificial media, and absence of demonstrable nucleus. None of the spirochetes forms cysts or true endospores nor does any of them contain pigments, although some form granules of volutin, a substance commonly found in true bacteria. There are neither sexual differentiation nor sexual phenomena.

**Divisions of the Order Spirochaetales.**—A short review of the more important or interesting species may be divided into two divisions, the first including three wholly saprophytic genera (*Spirochaeta*, *Saprosira*, *Cristispira*) characterized, according to Noguchi,<sup>1</sup> by being resistant to the action of 10 percent solutions of bile salts, the second comprising three parasitic genera (*Tre-*



*ponema*, *Borrelia*, *Leptospira*) which are completely disintegrated by such solutions.

**Genus Spirochaeta.**—The name of this genus is derived from the name "Spirochaete," by which Ehrenberg, in 1833, designated a very large, spiral and flexible organism found free-living in stagnant water. This organism is now the type species of the genus *Spirochaeta* and is called *Spirochaeta plicatilis*. It is a slender spiral (0.5 microns in diameter) but often attains the relatively enormous length of 500 microns (0.5 mm.). There is a distinct, wavy, axial filament around which the protoplasm is coiled in rather close spirals. The ends are blunt. Large granules of volutin are present (Figs. 335 and 337). There are no septa or evidences of

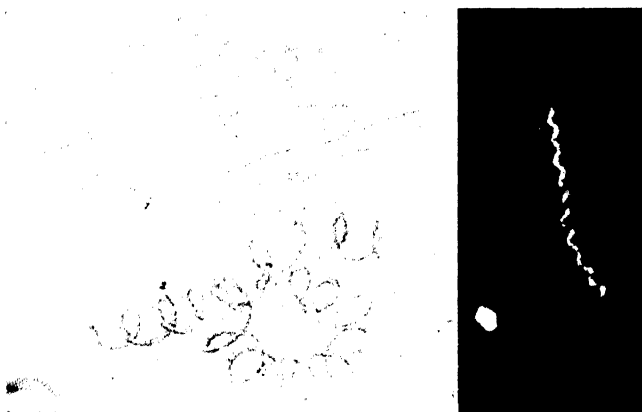


Fig. 337.—*Spirochaeta plicatilis*, or similar species. (Ehrenberg.)

a well differentiated cell membrane. Motility is by creeping movements. Specimens may be found in sewage and stagnant water and are cultivable in such material under anaerobic conditions, provided hydrogen sulfide be present. Pure cultures have not been obtained. They are entirely harmless as far as is known. Several species besides *S. plicatilis* have been described in this genus.

**Genus Saprospira.**—This genus resembles the foregoing in that the organisms are found free-living in water, have blunt ends, and are curved and wavy. They are not, in a living state, actually spiral like the *Spirochaeta*. When dead, they may be contracted to a spiral form. The organisms are thicker than *Spirochaeta plicatilis*, having a diameter of about 1 micron, but are shorter, seldom exceeding 85 microns in length. Some are much smaller than this.

Saprospiras possess no demonstrable axial filament but have a septate or chambered protoplasm, a distinct cell membrane and contain volutin granules. Some species are found in the intestinal tract of mollusks. The group is very little known. Saprospiras have never been cultivated.

**Genus Cristispira.**—In most respects the cristispiras resemble the saprospiras. They have a wavy arrangement, blunt ends and a septate protoplasm without axial filament (Figs. 335 and 338). The cell membrane is distinct. Motility is active.

There are two matters of especial interest connected with cristispiras. One is their habitat, which is restricted to the intestinal

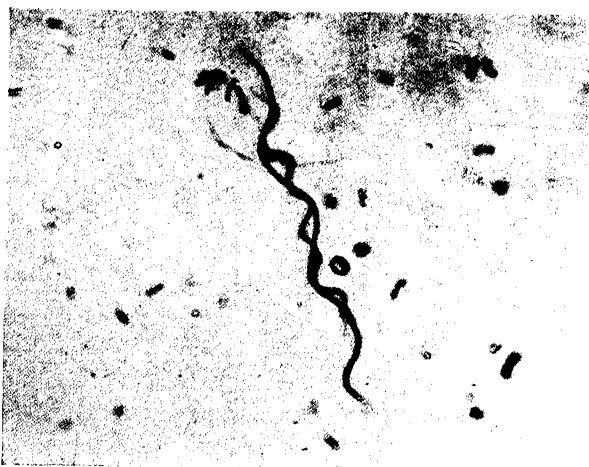


Fig. 338.—*Cristispira balbianii*, showing crista. (Hideyo Noguchi.)

tract of oysters and other lamellibranchs or to a hyaline structure, called the *crystalline style*, in the esophagus of these mollusks. The other is the presence of a sort of keel, consisting of parallel, flagellum-like fibrils, which winds spirally about the organism, one edge thickened and free, the other thinner and attached to the cell proper. This structure is suggestive of the keel-like undulating membrane seen in one genus of pathogenic protozoa, the trypanosomes (see page 788). The generic name is derived from this membrane or *crista*. The organisms are entirely harmless. None of the cristispiras has ever been cultivated.

**Genus Cytophaga.**—This genus, at present tentatively included with the spirochetes on a morphological basis, should probably be

transferred to the order Myxobacteriales.<sup>2</sup> These organisms inhabit the soil and sea waters and are of interest because they are among the most active aerobic, cellulose-decomposing bacteria and because they exhibit several other curious phenomena. The cells are from 3 to 8 microns long and 0.5 to 1 micron in diameter (Fig. 339). They may have rounded or pointed tips. Spiral forms are frequently seen in stained preparations but some authorities believe these also result from distortion due to drying and staining. Cytophagas are stained with difficulty, like spirochetes, but are gram-negative. Giemsa's stain followed by tannin solution may be used.



Fig. 339.—*C. rubra*. Attacking filter paper fiber. Winogradsky stain.  $\times 835$ . (R. Y. Stanier, Bacteriological Reviews, Vol. 6, Williams & Wilkins Co.)

These morphological properties and flexibility are the principal reasons for including the genus with the spirochetes.

Cytophagas are strict aerobes, growing well at temperatures between 20° and 30° C., prefer slightly alkaline media (pH 7.5), and are sensitive to acid.

Their motility is of an interesting type, consisting of creeping and flexing movements only in contact with some fixed or solid surface. Sometimes they swing by one end from a fixed surface and oscillate like a pendulum. No flagella have been demonstrated.

For cultivation of soil cytophagas an initial enrichment in a solution containing inorganic substances for all except carbon requirement will suffice. Such a solution may consist of

KNO <sub>3</sub> .....	0.1	gm.
K <sub>2</sub> HPO <sub>4</sub> .....	0.1	gm.
MgSO <sub>4</sub> .....	0.02	gm.
CaCl <sub>2</sub> .....	0.01	gm.
FeCl <sub>3</sub> .....	0.002	gm.
Water.....	100.0	cc.
pH to 7.5		

To this are added some bits of filter paper as a source of carbon. These organisms can utilize cellulose as a source of energy, and, in fact, can utilize few other carbon sources, exceptions being cellobiose and glucose. A few species may attack mannite and xylose.

As shown by Stanier<sup>2</sup> poured agar plates of this mineral composition may be used with glucose or *finely shredded* filter paper. A curious fact is that the glucose must be sterilized by filtration, since heating, even to 50° C., induces some obscure but very potent unfavorable change to occur in it. Peptone must be used for nitrogen sources when studying marine forms.

The colonies of soil forms appear at first on cellulose or glucose-mineral agar as yellow, orange or pink discs that have a mucoid appearance due to the formation of a slimy, carbohydrate, gum-like material. The cells tend to creep outward away from the colony, forming mucoid outgrowths that spread more or less extensively and rapidly, depending on the species. The phenomenon is referred to as swarming. This characteristic is well exemplified in the order of Myxobacterales. Swarming is particularly well developed in marine cytophagas. It is interesting that these forms are obligately halophilic, failing to grow well in saline concentrations of less than about 2 percent. If the seas were the original habitat of living forms, it may be that halophilic marine cytophagas are more primitive creatures than those adapted to waters of much lesser salinity and to soil.

Some species of *Cytophaga* produce dormant bodies called microcysts or sporoids. These develop by a shortening and rounding up of the whole cell. They are surrounded by a mucilaginous sheath (Fig. 340). Microcysts are unlike the endospores of true bacteria in having little greater resistance to heat than the vegetative cell. They resemble rather closely the microcysts of the slime bacteria (Myxobacterales) in formation, resistance and function. Indeed, the cytophagas, especially those species which form microcysts, might well be classed with the Myxobacterales because of their swarming habit, flexibility, creeping motility and microcyst formation. Obviously the way of the taxonomist is hard—but fascinating!

**Genus Treponema.**—Treponemas are much smaller than the organisms of any of the spirochetes so far discussed (except *Cytophaga* which is probably not a true spirochete); they are often exceedingly slender (0.25 microns to 0.4 microns in diameter), seldom exceeding a length of about 15 microns and are often shorter than this. Their size is therefore comparable with that of the Eubacteriales. Their cell structure is much less perfectly demonstrable than is that of the four preceding genera. The organisms have neither crista nor septa and the presence of an axial filament is a matter of question. However, by means of plasmolysis experiments, both axial filament and periplast are

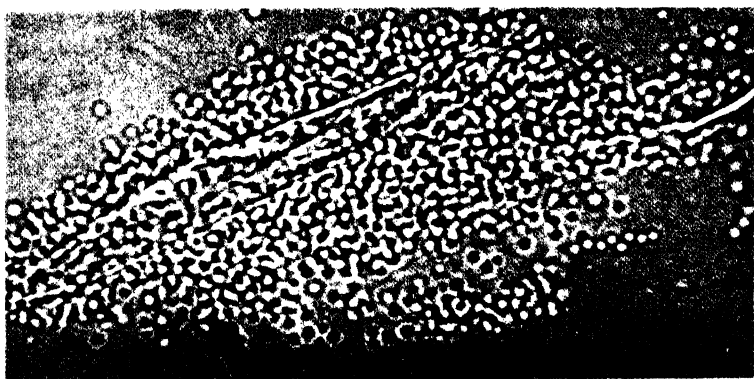


Fig. 340.—*S. myxococcoides*. Examples of the typical "Mikrokokkenschleim" resulting from microcyst formation on attacked cellulose fibers. The material was taken from an impure culture on filter paper after five days at 30° C.; photographed in the living state. Magnification  $\times 940$  approx. (R. Y. Stanier, Bacteriological Reviews, Vol. 6, Williams & Wilkins Co.)

said to have been demonstrated, but the former is not shown by the electron microscope<sup>3</sup> (Fig. 336).

The eight to fourteen spirals found in treponemas are close and regular unless the protoplasmic contractions change them. The ends of the organisms are drawn out into extremely fine fibrils which may represent either prolongations of the axial filament or merely remnants of the fine thread of cytoplasm connecting the organisms before fission is complete. These terminal fibrils have no function in the motility of the organism. Motion depends on flagella in some species and probably also the propeller-like action of the spirals when the treponemas rotate.

Treponemas are not easily stained. Indeed, the first treponema

to be described, that causing syphilis (Schaudinn, 1905), was named *Treponema pallidum* because of its pale appearance when subjected to stains. Other methods are, therefore, used to demonstrate them microscopically. One is the method of negative staining; another, the darkfield apparatus (see Chapter 3); and a third is termed "silver impregnation." For this, several methods are available, a useful one that of Fontana.\* In this method ammoniacal solutions of silver salts are allowed to penetrate the cells and metallic silver is then precipitated there by means of a reducing solution. The organisms appear black against a yellowish background (Fig. 341).



Fig. 341.—*Treponemas* of yaws in tissues stained by silver impregnation. (Ferris and Turner.)

*T. pallidum* is a relatively fragile organism. It has never been cultivated in artificial media although this feat has been claimed by some. The organisms survive, under ordinary circumstances, for only very short periods outside of the tissues of man or experimentally infected animals. Hence nonvenereal infection of man is rare. Turner and his associates have shown that, when quickly frozen and maintained at  $-76^{\circ}\text{C}$ . by means of solid carbon dioxide, syphilis spirochetes, as well as other organisms, will remain viable and fully infectious for years.<sup>4</sup>

The organisms do not long survive ordinary drying.<sup>5</sup> In citrated blood stored in "blood banks" they quickly die out.<sup>6</sup>

\* Fontana's silver stain for spirochetes:

Solution A		
Tannic acid. . . . .		5 gm.
Distilled water. . . . .		100 cc.
Solution B		
Silver nitrate. . . . .		5 gm.
Distilled water. . . . .		100 cc.

To 90 cc. of solution B slowly add concentrated ammonium hydroxide till a brown precipitate forms and *just* re-dissolves. Then add, very cautiously, more of solution B till the fluid shows a faint, permanent cloudiness. This "keeps" well.

Flood smear with solution A and steam for about 30 seconds. Wash with water and flood with solution B, steaming for about 30 seconds. Wash and blot.

**Syphilis.**—Syphilis, caused by *Treponema pallidum*, is primarily a venereal disease, *i.e.*, is transmitted chiefly by sexual intercourse. When so transmitted and when it develops typically it begins, within a month or less after exposure, as a small ulceration on the mucosal surface of the genitalia. The ulcer increases in size, becoming rather hard and flat. Upon removal of the crust a serous fluid oozes from the surface and, upon examination with a darkfield apparatus, may be found swarming with *Treponema pallidum*. When syphilis is acquired through kissing, the chancre may occur on the lip. This ulcer, oral or genital, is spoken of as a “primary lesion” or *hard chancre* (pronounced shank’er) (Fig. 342). It tends to heal spontaneously due to the development of antibodies<sup>7</sup> after two or three weeks and the victim may believe himself cured. Many proprietors of quack remedies and vicious charlatans preying upon the fears and ignorance of their fellows have thrived upon this fallacy.<sup>8</sup>



Fig. 342.—Chancre of the lip. (Keidel.)

What really happens is that by this time a certain degree of immunity develops. The treponemas have *long since migrated* from the primary lesion and been carried all over the body, localizing in various organs, particularly the liver, spleen, walls of arteries, heart, brain, skin and mucosal surfaces, setting up “secondary lesions” after two to four months. When situated on the skin these appear as red blotches or an extensive rash and may be very infectious (Fig. 343). They may occur on any part of the body and teem with treponemas. Infectious white patches may also appear in the mouth and genitalia. In such conditions of the mouth kissing of other persons results in infection of the lips, tongue or gums of the latter. The teeth may loosen and come out, as well as the hair. The latter becomes patchy looking and is often spoken of as “moth-eaten hair.” After a time (weeks or months) these outwardly visible, *secondary* lesions slowly disappear in great part and the patient may again believe himself cured. Sometimes spontaneous cure actually does occur.

The treponemas, however, usually slowly cause extensive *tertiary* lesions called *gummata* in various internal organs and also on the skin. The liver becomes damaged and scarred (syphilitic *cirrhosis* of the liver) and bulges appear in the aorta where lesions in the layers of the vessel have weakened it. These bulges are called *aneurysms*. When they burst, death from hemorrhage often ensues. Gummata also occur in any of the bones (Fig. 344).

The treponemas also damage the brain and spinal cord. Various centers are slowly destroyed and characteristic forms of insanity and paralysis result. Death follows, sometimes after a period of many years.



Fig. 343.—Secondary lesions of syphilis. (After Chesney, Turner and Halley.)

This picture of syphilis is not pleasant and, indeed, the disease is one of the most insidious and dangerous. In 1943, nearly 13,000 persons died of this disease in the United States, but many cases existed which did not come to the attention of the recorder.

As in the case of gonorrhea, prostitution is the chief means by which syphilis is spread.<sup>9</sup> In spite of renewed efforts by federal, state and local authorities to educate the public to the dangers of syphilis and to enlist the aid of legislatures, medical and civic authorities in preventing its spread, many new cases appear each year. However, syphilis is no longer a secret and sneaking enemy of the shadows, but it has been brought into the light by public



opinion, by open, matter-of-fact discussion in newspapers and public meetings. The medical officers of the armed forces have done much in the campaign of education and prevention, and greater enlightenment on this subject is one of the many benefits to the country as a whole from military service.

Aside from prophylactic measures which need not be discussed here, it may be controlled to some extent by measures directed against prostitution and by early treatment, but the treatment is

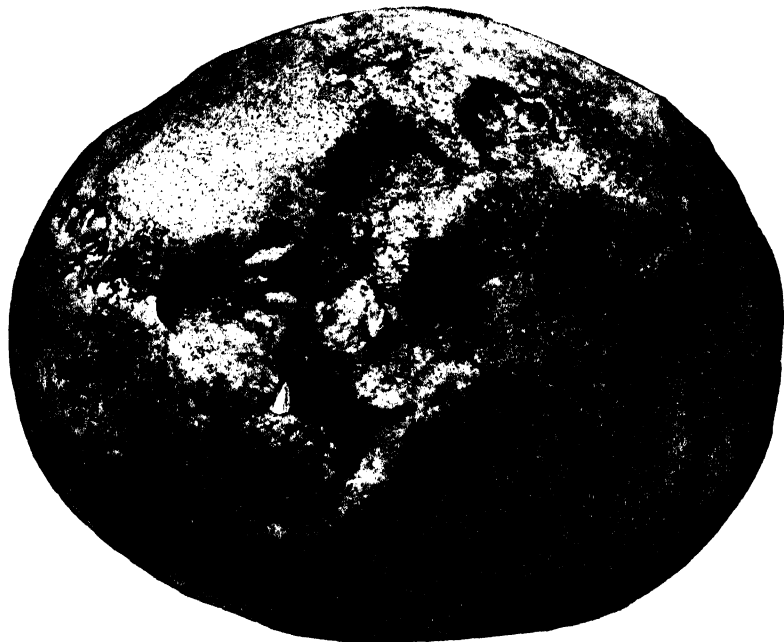


Fig. 344.—Old syphilitic erosion of the skull. The margins in this case are smoothed off by a healing process. (MacCallum.)

long, expensive, painful and involves arsenic, a dangerous poison. A short-time, intensive mode of arsenical treatment, although given much newspaper publicity, is still in the experimental stage. The use of penicillin has given dramatic results.<sup>10</sup>

Possibly Americans who have seen service in North Africa may have had occasion to observe an interesting variant of syphilis called *bejel*. It has been described by Hudson.<sup>11</sup> The lesions characteristically occur on the lips. The causative agent may differ from *T. pallidum*.

The diagnosis of *syphilis*, after the disappearance of the primary lesion in which the spirochetes are demonstrable microscopically by means of the darkfield apparatus, is made by means of the Wassermann test or a precipitin test such as the Kahn, Eagle or Kline test (see page 304).

**Yaws and *Treponema pertenue*.**—Yaws is an ulcerative disease, common in, and restricted to, tropical countries. The earliest lesions usually appear on the skin of the feet, legs and arms (Fig. 345). It is not a venereal disease, evidence obtained by Turner and



Fig. 345.—Yaws. Showing extensive spread of crusted lesions (Tonga Islands). (MacCallum.)

Kumm indicating that it may be insect-borne.<sup>12</sup> The causative organism, *Treponema pertenue*, is morphologically indistinguishable from *T. pallidum*. Patients with yaws give a positive Wassermann test and respond to the same treatment (salvarsan, arsphenamine, etc.). Yaws is not so dangerous a disease as syphilis but causes very extensive syphilis-like lesions nevertheless. Natives of the tropics are said to recover from it without treatment.

Yaws illustrates a very interesting relationship of one spirochetal disease to another. An attack of yaws (as a rule) apparently confers complete immunity to syphilis. Does yaws represent merely a different clinical manifestation of

syphilis; is it a different disease; or is *Treponema pertenue* a modified type or variant of *T. pallidum*, changed by contact with a different race of people so that it has lost virulence, much as smallpox virus loses virulence for man on contact with cattle? At present, there is no definite answer to these questions, but Ferris and Turner<sup>13</sup> have shown that the spirochetes of yaws produce, in rabbits, lesions which are readily distinguishable from those produced in rabbits by *T. pallidum*, indicating that there are definite differences between the organisms. Furthermore Turner and McLeod<sup>14</sup> showed that rabbits having received intra-

testicular inoculations of *T. pertenue*, *T. cuniculi* or *T. pallidum* were later found to have considerable resistance to each of the heterologous species. As stated by these authors: "The ability of an organism (*T. cuniculi*), causing a natural disease of rabbits, to produce an increased resistance to syphilis is of particular interest." These observations are illustrative of the general proposition that closely related species often contain antigens in common which may be of the greatest value in studying all types of taxonomic relationships as well as in developing new methods for immunization against disease.

**Genus *Borrelia*.**—While there are differences in morphology, pathological action and resistance to external conditions, the borrelias resemble the treponemas closely in many respects, such as cell structure and staining peculiarities. However, many borrelias can be stained readily by Gram's method or by means of a special polychrome stain made by mixing eosin and methylene blue (Jenner's stain, Wright's stain\*). Morphologically, they often differ in having a less definite spiral form, being more wavy and open (Fig. 346). They are also somewhat thicker and coarser-looking than the treponemas. Cultivation has been accomplished although it is not very satisfactory. The culture medium devised by Noguchi is said to give good results. This consists of tall narrow tubes containing deep (15 cm.) columns of serum or ascitic fluid mixed with water and containing bits of sterile rabbit liver, kidney or testis. Agar is added in 0.2 per cent concentration making a soft, mushy mixture.

Commensal species of *Borrelia* occur, often in large numbers, in the mouth (*Borrelia macrodentium*, *B. microdentium*) and on the external genitalia (*B. refringens*). Some of these so closely resemble *Treponema pallidum* in appearance as to create confusion at times in the diagnosis of syphilis by microscopic methods. They appear to

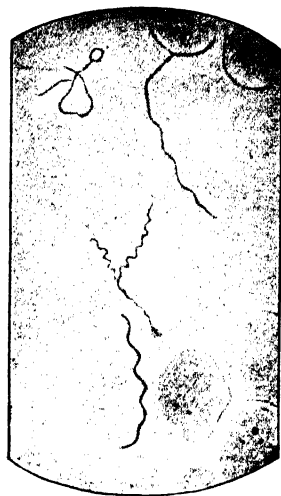


Fig. 346.—The two spirochetes in the center are *Treponema pallidum*, the three others, *Borrelia refringens*. (Schaudinn and Hoffmann.)

\* These stains are commonly used for staining blood films to demonstrate the protozoa of malaria.

be harmless. They are sometimes classed as *Treponema*. Leeuwenhoek probably was the first to observe these. The majority of the pathogenic species, like *B. recurrentis*, are blood parasites causing fever of a relapsing nature.

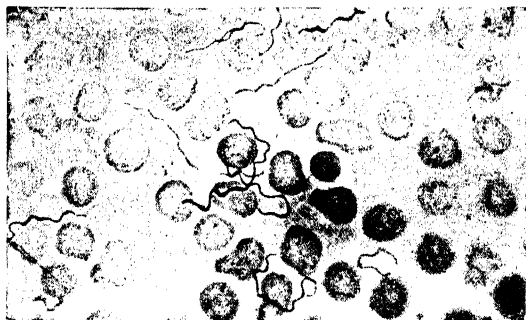


Fig. 347.—*Borrelia duttoni* of African tick fever in a blood smear ( $\times 900$ ). (From preparation furnished by Dr. G. N. Calkins.) (Zinsser, Bayne-Jones, "Text-book of Bacteriology," D. Appleton-Century Co., publishers.)

The blood-inhabiting borrelias (Fig. 347) differ from the treponemas in being transmissible from one person to another by certain insects (*Ornithodoros* ticks or lice), in which they survive for considerable periods although often in an apparently unrecognizable form so minute that they will pass porcelain filters. This lends support to the view that they pass into an invisible granular stage which is filterable. Filtered blood of relapsing-fever patients also transmits the disease.



Fig. 348.—African relapsing fever tick, *Ornithodoros moubata*.  $\times 3$ . (From Herms, "Medical and Veterinary Entomology," by permission of The Macmillan Co., publishers.)

**Relapsing Fever.**—Relapsing fever is characterized, as the name indicates, by a series of relapses after the first fever subsides, which it often does very suddenly. The disease is found in America, transmitted by lice and ticks, in Central Europe, where it is transmitted by lice, and in tropical and subtropical countries where it is tick-borne (Fig. 348).

The organisms are found in the blood of patients during the febrile periods, but during the afebrile interludes seem to retreat to

internal foci in the liver, spleen and lymphatics. Young rats can readily be infected with febrile blood. One of the best known species, *B. recurrentis*, was discovered in the blood of European relapsing fever patients by Obermeier in 1867. Other species, found in different places and named for different workers, are *B. duttonii* (Africa), *B. novyi* (America), and *B. carteri* (India). The differentiation between these species is made on bases of host specificity, serology, and geographical location. The tropical species are more dangerous than the European and American, the latter rarely causing fatal disease (2 percent fatality).



Fig. 349.—Vincent's angina. Stained smear showing fusiform bacilli and spirochaetal forms. ( $\times 900$ .) (Todd and Sanford.)

**Premunition.**—As immunity develops, the relapses recur less and less frequently, but chronic infection persists for a long time, maintaining immunity by *premunition*, i.e., presence of the organism in the body. The relapses probably represent the oscillations of a balance between the virulence of the organisms and the attempts of the host to become immune. The febrile attacks decrease as equilibrium is established.

Treatment of these *Borrelia* infections is similar to that used in syphilis and yaws but only one or two injections of arsenical drug are needed as a rule.

**Trench Mouth.**—Some anaerobic species of *Borrelia* are found in the mouth (notably *B. vincentii*) associated with ulcerative

conditions ("trench mouth," or *Vincent's angina*). They may be seen readily in gram-stained smears from such conditions, mixed with fusiform bacilli (Fig. 349). The name "trench mouth" originated in the frequent occurrence of outbreaks of the disease in soldiers in trenches during World War I. It is presumably transmitted by unclean eating utensils and other articles which carry saliva directly from mouth to mouth. It may be associated with dietary deficiencies.

**Genus *Leptospira*.**—The organisms of the genus *Leptospira* are the smallest of the spirochetes. Their spirals are so fine and so closely wound that, when observed in the darkfield, only the outer curves of the spirals are seen and the organisms appear like



Fig. 350.—*Leptospira icterohaemorrhagiae*. Appearance of organisms in the darkfield. (Zinsser, Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

strings of minute, illuminated beads. They are said to have a very fine axial filament and terminal filaments. The leptospiras are further characterized by being bent into a hook at one or both ends (Fig. 350). Their motion consists of a writhing and flexing movement and a rapid rotation around the long axis. Their progression from place to place is rapid and cannot be readily explained. Possibly the electron microscope may reveal flagella, as it presumably has done for treponemas. Microscopically, they are demonstrable by silver impregnation, India ink and darkfield methods. They may be cultivated readily in semisolid (0.2 percent) infusion agar containing 10 percent rabbit serum and about 90 percent physiological saline solution. The pres-

ence of the soft agar is highly beneficial to these organisms. They are microaerophilic, growing best about 1 cm. below the surface of the medium in a narrow zone.

Leptospiras are chiefly saprophytic, aquatic organisms, which are found in river and lake water, in sewage, and in the sea. Occasionally, they are found in the normal mouth. As shown by Bauer and Sawyer<sup>15</sup> and others, they may be cultivated in pure culture from some pond or swamp waters by the simple procedure of passing the water through a porcelain or kieselguhr filter (through which they readily pass), and then adding (aseptically) sterile serum, salt and melted agar to yield the proportions given above.

Leptospiras may frequently be demonstrated in river, lake, or well water by adding to a liter of it the yolks of three eggs or a few grams of fecal suspension and incubating in a broad, flat dish at room temperature.

*Relation between Pathogenic and Saprophytic Leptospiras.*—Leptospiras (*Leptospira icterohaemorrhagiae*) morphologically and culturally indistinguishable from the harmless species found in nature occur in the blood and urine of patients suffering from Weil's disease (*hemorrhagic jaundice*). Several other clinically similar conditions are due to other species. For the present we may only speculate on the relationships between the saprophytic and the pathogenic forms. Authorities disagree as to whether the pathogenic species are derived from the saprophytes through a process of adaptation to life in a host by passage from one host to another with resulting increases in virulence; or whether the two are entirely separate kinds of leptospiras. There is experimental evidence on each side.

For one thing, the pathogenic types can survive free in wet soil and in water for long periods and, since they can also infect rats, may be spread about by the urine of these ubiquitous animals and thus appear to be "free-living" water forms when their source is not known. Thus, rat-injection experiments with supposed "water leptospiras," in which infection is obtained, could easily be vitiated by unsuspected contamination of the water with pathogenic strains from rats.

**Weil's Disease.**—This disease is quite widespread. It is common among persons who spend much time in wet, poorly drained situations such as badly constructed mines, wet trenches during war, and the bilge of river and lake boats, especially if rats abound there. In the last year or so it has been found with increasing frequency in the United States. Ward and Turner,<sup>18</sup> in a survey of Baltimore poultry-dressing, meat and related establishments, found much evidence of it among employees (17.3 percent). Rats infested the premises. They also found active infection in persons using water from a well near an airplane factory in that city and demonstrated the leptospiras in the water. Rats were abundant around the well. As shown by Meyer and others,<sup>19-25</sup> dogs may likewise transmit this organism, as well as a similar species, *L. canicola* which produces fatal jaundice in them, to man. Clinically Weil's disease resembles yellow fever (a disease due to a filterable virus, see page 718) very closely. In well-defined cases there is an onset lasting from one to several days involving chill, muscular pains,

headache, fever, nausea and vomiting. The kidneys are affected, much albumin appearing in the urine. Other organs also are damaged, the liver so much so that marked jaundice appears, giving a definitely yellow color to the skin which is characteristic of this disease and of yellow fever. In the severer cases, hemorrhages into the stomach and intestines occur, resulting in a dark-colored vomitus due to the action of the gastric juice on the blood ("black vomit"). Similar symptoms and signs are found in yellow fever.

The disease has frequently been confused with yellow fever in areas where yellow fever was endemic and has produced, at times, a high mortality.



Fig. 351.—*Leptospira* undergoing lysis in Pfeiffer's phenomenon ( $\times 900$ ).

*Leptospira icterohaemorrhagiae* occurs in the blood and urine of human beings ill with the disease and may be cultivated from them by methods given above. It is readily transmitted from man to man by polluted water and is thought to get into the body also by way of the feet when one remains long in infected water. It is evident that rats may transmit the disease, polluting sluggish streams, mines, ships, trenches, etc.<sup>16-22</sup> As pointed out above, *L. canicola* causes a similar disease in dogs and is found in the urine of these animals. It may infect man.<sup>23-25</sup>

*Immunity in Weil's Disease; Pfeiffer's Reaction.*—Shortly after the onset of infection with *Leptospira icterohaemorrhagiae*, lytic antibodies begin to appear in the blood stream. These increase in concentration until the serum of the person is capable of protecting



him against large doses of the leptospiras. The agglutinin titer increases markedly and is useful in diagnosis.<sup>26</sup>

The phenomenon of cytolysis may be beautifully illustrated with such serum. A drop of culture of *Leptospira icterohaemorrhagiae* is mixed with some of the patient's serum which has been heated at 56° C. for thirty minutes to destroy the complement in it. The leptospiras remain unchanged in any visible way. One can then demonstrate that they have been sensitized by the *lytic* antibodies in the serum. When fresh, complement-containing serum of some normal animal (e.g., guinea pig) is added, the leptospiras may be seen, by means of a "darkfield" apparatus, to swell, granulate, and undergo various distortions and finally to disappear (Fig. 351).

Pfeiffer, while studying cholera, was first to describe this cytolytic type of reaction and the phenomenon bears his name. Instead of applying complement in the form of fresh serum, however, he injected his sensitized cholera vibrios into the peritoneal cavity of normal guinea pigs where the complement worked the same as in a test tube. He made his observations on the progress of lysis by periodically puncturing the peritoneal cavity and withdrawing a tiny drop of the fluid for microscopic examination.

Active, artificial immunization is said to be effective in inducing potent antibody production against *Leptospira icterohaemorrhagiae*.<sup>27</sup>

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## CHAPTER 42

### THE VIRUSES\*

IN 1891 bacteria were the smallest, simplest and lowest forms of life known. Physiologically and structurally they were viewed as the boundary between the living and the inert. Investigators of that time felt that they had probed the depths of the mystery of life and discovered its extreme lower limit with respect to size and simplicity of organization. Even with microscopes capable of magnifying 3000 to 4000 diameters (the ordinary student's microscope has a maximum of about 900 diameters), no smaller living creatures were to be found anywhere. Scientists had reached the "jumping-off place" of smallness. Beyond the last visible bacterium all space, as far as their information went, was void of life. Yet many times the "void and empty" fluids through which their searching lenses swept, teemed with billions of living particles which escaped their vision and their knowledge.

**First Demonstration of a Filterable Infectious Agent.**—It was in 1892 that the existence of such living things was even vaguely suspected, after Iwanowski demonstrated that a disease of the tobacco plant called "tobacco mosaic" could be transmitted to healthy plants by sap from the diseased plant after it had been passed through filters of porcelain so fine as to remove completely all visible or cultivable bacteria.<sup>1,1a</sup> This discovery was later confirmed by others, the observations were extended to other diseases of both plants and animals, and today we realize that the discovery of Iwanowski opened the door to the *world of the ultramicroscopic*, much as Leeuwenhoek's discoveries opened the door to the world of the microscopic. Today many agents of disease related to the one discovered in 1892 are well known. They are designated, because of the invisibility of most of them by ordinary microscopes and their filterability through fine porcelain filters, as *filterable* or *ultramicroscopic viruses*. It is also important to note that our knowledge of them is based on the fact that they produce disease. No saprophytic viruses are known. They present to the biologist, the chemist and the philosopher one of the greatest mysteries, one of the most fascinating problems and one of the outstanding challenges of the century. In the limited

\* *Virus* is a general term meaning, strictly, a slimy substance. The word is restricted in bacteriology to the type of disease agents under discussion.

space here available, we can present only a few main facts concerning these mysterious organisms.

**Properties of Viruses.**—*Visibility.*—The most obvious properties of the ultramicroscopic viruses may be summarized very briefly. First is their *invisibility with ordinary microscopes*. No one has *with certainty* seen filterable virus particles. They cannot be demonstrated, *beyond quibble*, with any optical apparatus at present



Fig. 352.—Electron micrograph showing molecules of tobacco-mosaic virus.  $\times 34,000$ . (Stanley and Anderson, J. Biol. Chem., Vol. 139.)

extant. Hence the term *ultramicroscopic*. However, it seems probable that certain crystals and certain granules, to be described, are actually viruses. The electron microscope is opening a new field for the investigation of viruses. It has been possible to make shadow-graphs showing particles of definite, uniform size and shape under circumstances, and in infectious fluids, which leave the clear impression, and moral assurance, that these particles are

indeed the hitherto unseen viruses themselves (Figs. 352, 353, 354).<sup>2</sup> There is a wide gulf between moral assurance and absolute proof. Nevertheless, historical progress sometimes results from an expedient assumption of identity, a process which we may refer to as *bridging factual hiatus by hypothesis*. Such bridges are often built under intellectual verbal fire!

**Elementary Bodies.**—Several virus diseases are always accompanied by the accumulation in the body tissues, fluids, blood or lymph, of myriads of small refractile granules, approximating the smallest bacteria in size, and visible with ordinary microscopes. They are often called elementary bodies.<sup>2a</sup> Their presence and number fluctuate with the presence of the disease and the infectivity of the fluid containing them. They may be stained by appropriate methods (Giemsa, etc.). Separated by centrifugation and washed free of other substances they retain their infectivity. Their very size and form, which vary greatly, may be characteristic of a given disease. Further, they may be agglutinated by specific immune sera and robbed of their infectivity by such sera. But their actual identity is not known with absolute certainty. Granules of degenerated protein or other matter are often present in material containing viruses, and no sure means of distinction has been described. Viruses tend to attach themselves (by adsorption) to minute particles of various kinds so that one can never be sure, when he sees such granules, whether they are virus particles or merely inert particles on which virus may have been adsorbed. Immune sera might agglutinate such particles and neutralize the virus adherent to them. Further, many viruses develop in tissues without the appearance of demonstrable granules. A cubic centimeter of serum of animals infected with yellow fever may be perfectly limpid to the eye, and perfectly free from any visible abnormal appearance or substance whatever, yet contain sufficient virus so that one millionth part or less of the cubic centimeter can cause a rapidly fatal attack of yellow fever. There is not even any abnormality in refractive index of the serum.<sup>3</sup> This point suggests that some virus particles approach the size of molecules or may be invisible merely because they have the same refractive index as the fluid in which they are suspended.

These apparently contradictory views may result from the fact that some viruses are larger than others, so that while some are beyond the limit of visibility, others, as the Paschen bodies of small-pox, are within the range of ordinary microscopic vision.

One of the most interesting series of observations in this connec-

tion is that of Stanley<sup>2</sup> and others on the crystalline nature of the virus of plant diseases, notably tobacco mosaic disease and "bushy stunt." The crystals are obtained from infectious tissue juice by precipitation methods. The crystals seem to have all of the essential properties of the virus and appear to be nucleoprotein in nature. They are widely accepted, as the actual virus itself (Figs. 353 and 354). No virus of animal disease has been obtained in such form and it seems that plant viruses and animal viruses differ in the fundamental respect that animal viruses are not crystallizable and are much more unstable and susceptible to injury by chemical manipulation.<sup>4</sup>

**Cultivation.**—A second characteristic of viruses is their *refusal to multiply on artificial media*. No one has ever succeeded in produc-



Fig. 353.—Crystalline protein from tobacco mosaic. Crystals indicate chemical purity. The chemically pure substance of these crystals is said to be at the same time a living entity.  $\times 400$ . (W. M. Stanley.)

ing satisfactory evidence that viruses will grow in *nonliving* pabulum. Contrasted with this, however, is the case with which many viruses may be made to multiply in *living cells*. It is not excessively difficult to prepare cultures of cells of the body or cells of the embryos of chicks, and the like, and to infect these test-tube cultures with viruses. Or, one may proceed more directly and inoculate embryonic chicks *in ovum*. Two important adaptations of these methods of cultivation are those of Li and Rivers<sup>5</sup> and of Goodpasture.<sup>6</sup> Plant virus may be propagated in root-tip cultures. (See section on methods of cultivation, page 161.)

These methods require the most expert skill and absolutely aseptic technic because contamination with bacteria kills many viruses. Sulfonamid drugs may be used in some tissue cultures to

prevent the growth of contaminating bacteria.<sup>7</sup> *In the absence of living cells the viruses cannot multiply.* The scientist who devises a means of cultivating any true virus on nonliving medium may become the Leeuwenhoek or the Pasteur or Madame Curie of that day!

**Filterability.**—A third distinctive property of the viruses is their ability to pass through porcelain or other filters which hold back the

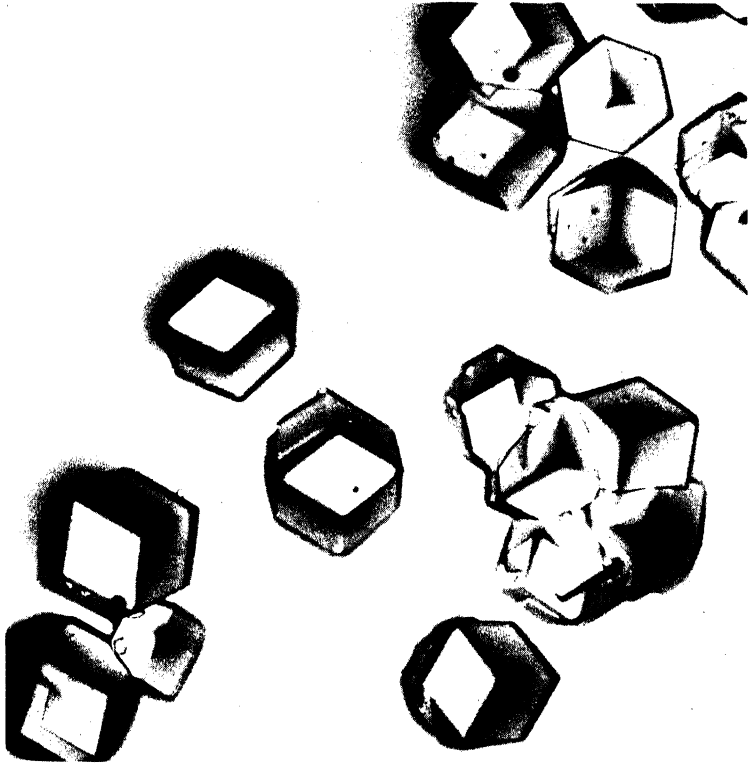


Fig. 354.—Crystalline bushy stunt virus.  $\times 224$ . (Stanley, J. Biol. Chem., Vol. 135.)

*smallest known bacteria.* This is to be expected of their small size. It is probable, however, that minute size is not the only property which enables them to pass filters, and that forces active at surfaces also play a rôle. Some viruses are filterable only with difficulty and under special conditions. The agents of vaccinia and rabies are of this type.

*Mechanism of Filtration.*—If the student will recall his studies of physics, he will remember that nearly all very minute particles, when suspended in watery fluids, acquire an electrical charge. Bacteria have negative electrical charges. The particles of material composing porcelain or kieselguhr filters have an opposite charge or at least a charge that is relatively less negative. Thus, when bacteria come into contact with such filters, the two unlike charges result in an attraction and the bacteria are firmly held so that the fluid in which they are suspended passes on through the filter without them. If they are small, and are hurried through the filter under high pressure, they may escape this electrical adsorption and appear in the filtrate. This has caused some workers to regard bacteria as having the ability to pass into growth phases similar to, or identical with, the ultramicroscopic viruses. Of course, the filters must be fairly fine in order to bring the bacteria close enough to the walls of the passages or tunnels through the filter so that they come within the very restricted sphere of influence of the mural electrical charges. Channels an inch in diameter would obviously defeat this purpose. Cracks in the filter would act in the same way and are a frequent source of error.

That filterability or nonfilterability of bacteria is, to a great extent, dependent upon electrical forces is made clear by the fact that if filters be composed of material having the same charge as the bacteria (plaster of paris), no adsorption of the bacteria occurs and they pass through without difficulty.<sup>8</sup>

Further, if a porcelain, bacteria-retaining filter be treated with serum, grease, heavy bacterial suspension, protein, or some substance capable of being adsorbed or, in other words, capable of neutralizing or rendering ineffective all the electrical charges and other surface forces on the filter particles, then bacteria will pass through without being adsorbed.<sup>9</sup> Thus, with respect to bacteria it seems that it is primarily electrical charges and other surface phenomena, and only secondarily size, that determines filterability through porcelain filters. This is not so clear when we come to consider viruses. These agents, like other minute particles should, theoretically, possess a negative charge and be adsorbed like bacteria. Contrary to theory, however, viruses are occasionally demonstrated to have positive charges.<sup>10</sup> Still contrary to theory, regardless of charge they all seem to possess the striking property of filterability. Obviously, we are not yet in possession of all the facts regarding filterability of minute organisms.



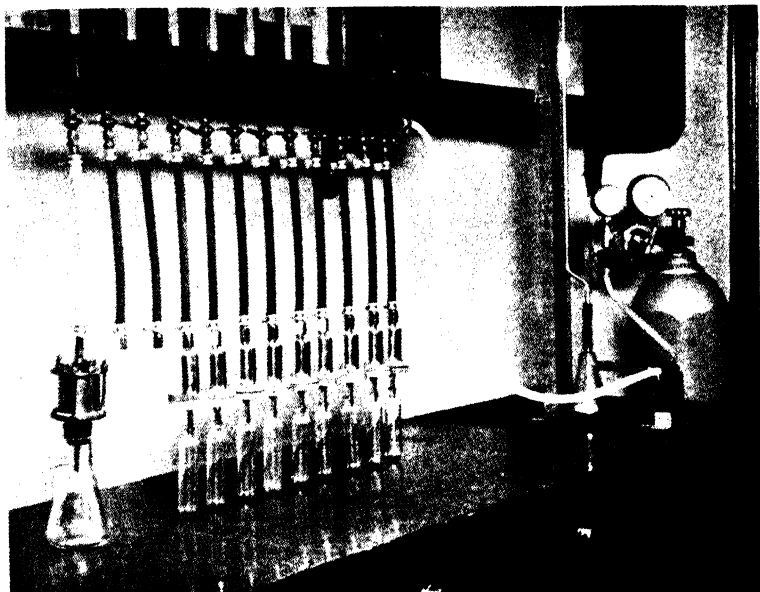


Fig. 355.—Series of collodion membrane filters of graded porosities for determining virus particle size. Note the pressure tank and mercury manometer for measuring the pressure applied. The virus suspension is held in the cylinder above each ampoule. The details of the filters are seen in Figure 356. (Bauer and Hughes, *Jour. Gen. Physiol.*)

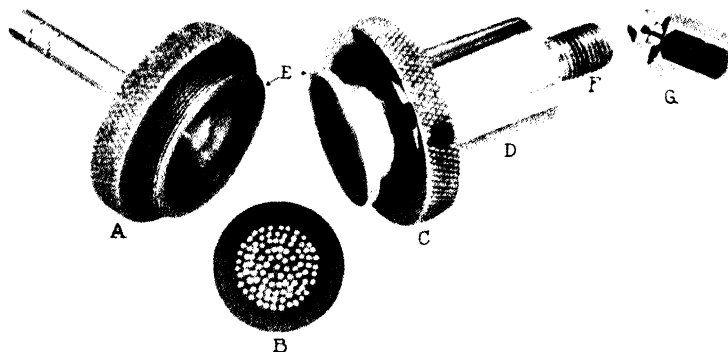


Fig. 356.—Detail of the filters shown in Figure 355. The collodion membrane, supported by a disk of filter paper, rests on B, which is set in A. Cylinder D is clamped onto A by collar C. The suspension is introduced through stem F. (After Bauer and Hughes.)

## COMPARATIVE SIZES OF VIRUSES

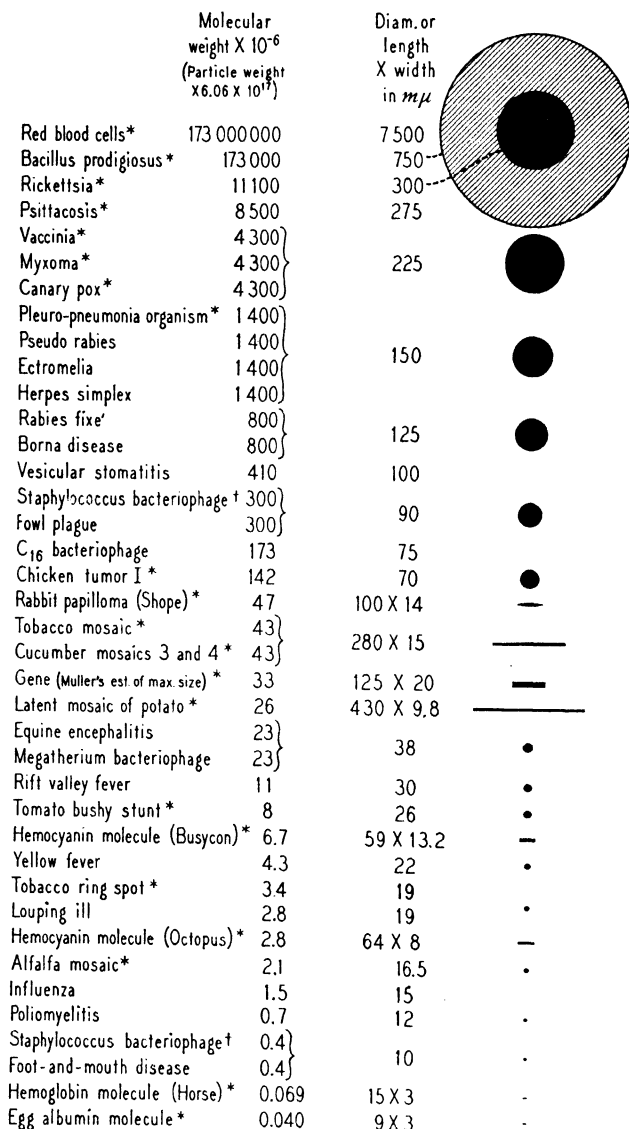


Fig. 357.

**Other Properties of Viruses.**—*Size.*—In the section on elementary bodies it was pointed out that the sizes of viruses may vary within rather wide limits. This has been proven by filtration through thin membranes of materials of varying degrees of porosity in which surface forces probably play a minor rôle. Thus, collodion membranes have a certain porosity. The degree of porosity (size and numbers of pores) can be regulated by controlling the density of the solutions of collodion used to prepare the membranes.<sup>11, 12</sup> By measuring the rate of flow of water at given pressures and temperatures through such membranes the diameter of the pores may be calculated. By passing a virus-containing fluid through a series of such porous membranes, starting with a coarse and ending with a fine filter, we may estimate the diameter of the virus particles by noting the coarseness of the filters which will pass them and the fineness of the filter required to withhold them (Figs. 355 and 356). The principle is the same as that on which a potato-grader or a gravel-sorter operates.

The data obtained by this means are of considerable interest. For example, it is possible to arrange different bacteria, viruses and protein molecules in a more or less accurate comparative series with respect to size as shown in Figure 357.

**Biochemical and Biophysical Data.**—In the last few years there have been intensive investigations of the physical and chemical properties of viruses.<sup>2, 4, 13, 14, 15</sup> The stability of plant viruses, permitting them to be precipitated out of suspensions by chemical means (such as 0.4 percent saturation with ammonium sulfate), adsorbed on celite or other finely divided material like kaolin or bone charcoal, eluted, reprecipitated, and otherwise manipulated for purification and concentration of workable quantities, without undue loss of activity, has made them a valuable subject for experimental research. It is not possible to give here all the data for all of the viruses, but examples are given to illustrate the general nature

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Fig. 357.—A chart showing the relative sizes of several selected viruses, including bacteriophages, as compared to those of red blood cells, *Bacillus prodigiosus*, rickettsia, pleuropneumonia organism, and protein molecules. The figures for size have been arbitrarily selected from data available in the literature. Particles known to be asymmetric are so indicated and the estimated length and width and the molecular weight in accordance with the asymmetry are given. In other cases where the particles are known or assumed to be spherical, the diameter and the molecular weight based on a sphere of density 1.3 are given. SYMBOLS: \* = evidence regarding shape available; † = large size from filtration and sedimentation of concentrated solutions and small size from diffusion of dilute solutions. (From Stanley and Knight, *Cold Spring Harbor Symposia on Quant. Biol.*, Vol. 9.)

of the new information being obtained and to indicate future trends in virus research.

*Plant Viruses.*—Stanley and his colleagues have obtained an extensive knowledge of plant viruses by means of highly purified preparations. Tobacco mosaic virus, one of the most widely studied plant viruses, occurs as needle-shaped liquid crystals (the paracrystalline state) which have been subjected to a variety of physical and chemical studies. It has been found, for example, that the crystals are doubly refractive, and possess positive electrical birefringence. The dimensions, as determined by calculation from sedimentation rates, specific viscosity and other physical data, are about  $15\mu\mu$  by 120 to  $280\mu\mu$ . Electron microscope findings give approximately the same figures. X-ray analyses yield information on molecular arrangements, etc. Chemically, the virus appears to consist entirely of nucleic acid of the yeast type and amino acids, with neither lipoids nor carbohydrates present. Amino acids in the nucleic acid of the virus include arginine, cysteine, glutamic acid, tryptophane, leucine, tyrosine and several others. Variations in the virus analogous to bacterial variations, and resulting in the production of new strains, are associated with definite changes in the amino acid content.

*Animal Viruses.*—Viruses less stable than that of tobacco mosaic, especially the animal viruses like those of influenza, yellow fever and poliomyelitis, are soon destroyed by the chemical and physical treatments used to purify and concentrate plant viruses. However, animal viruses have been purified and concentrated to a great extent, although none, with the possible exception of poliomyelitis virus, has been crystallized.

The most useful method of purifying and concentrating animal viruses for experimental purposes has been by means of the high speed centrifuge, driven by air turbines, on air bearings, in a vacuum. The process of purification and concentration consists in centrifuging first at relatively low speeds representing about 10,000 times gravitational force, discarding the relatively coarse sedimented impurities and then centrifuging the supernatant at a speed producing a centrifugal pressure equal to about 50,000 times the force of gravity. The sediment thus obtained contains much virus and is resuspended. The suspension is again subjected to alternate low and high speed centrifugation until a material with uniform specific gravity and other physical properties (*i.e.*, not a mixture) results. Such material may be subjected to various physi-

cal and chemical studies such as determinations of molecular weight, chemical composition, etc.

All viruses, plant and animal, so far isolated in this manner have been found to be proteins of very large molecular size and weight. For example, from sedimentation constants and other physical data the molecular weight of tobacco mosaic virus is estimated to be 43 million, that of influenza A virus about 650,000. Filtration and electron microscope experiments yield data concerning virus

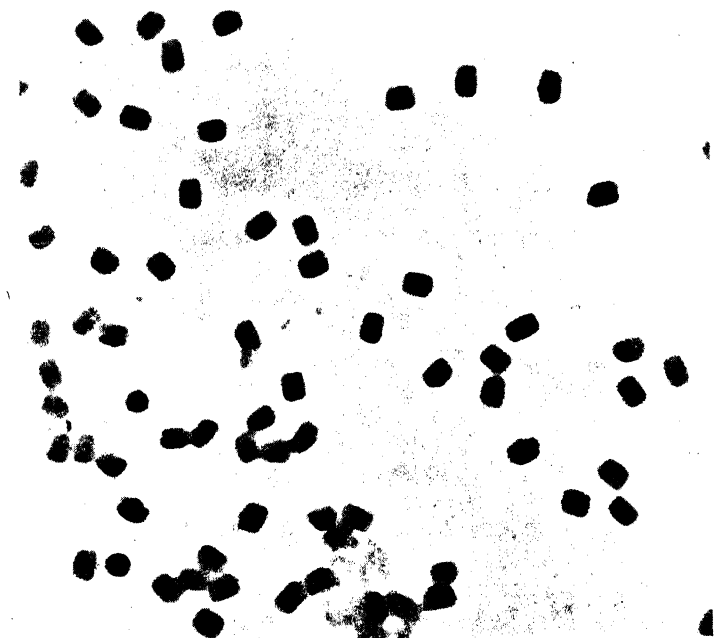


Fig. 358.—Vaccinia virus. Electron microscope picture of elementary bodies. ( $\times 20,600$ .) (Courtesy of R. C. A., Camden, N. J.)

particle size which agree, roughly, with the molecular weight data obtained with ultracentrifuge methods. The diameter of the influenza virus particle seems to be about  $100\mu\mu$ . Various other physical and chemical information of this general nature about several animal viruses is to be found in the very extensive literature on the subject.

*Role of the Electron Microscope.*—The electron microscope is playing a major role in the elucidation of the structure and size of viruses. For example, electronographs of the elementary bodies of

vaccinia (Fig. 358) show them to consist of roughly cubical packets each containing 5 block-like masses of unknown significance.<sup>2</sup> Vaccinia virus is one of the largest (around  $200\mu\mu$ ) and most stable of the animal viruses and is not so readily filterable as smaller viruses like those of poliomyelitis, yellow fever or influenza. Its concentration and purification by ultracentrifugation as previously described is therefore relatively simple.

*Antigenic Analysis.*—Antigenic analyses of viruses are proving of particular interest and importance. Tobacco mosaic virus, for example, seems to consist of but a single protein antigen, producing one antibody. On the other hand, animal viruses in general seem to consist of two or more antigens: the virus protein, which stimulates neutralizing antibody production, and a soluble antigen or mixture of antigens which appears to be a separate substance engendering the production of agglutinins, precipitins, etc. Vaccinia virus, for example, seems to consist of several antigenic substances, possibly five, corresponding to the structure shown by the electron microscope. One of these is heat labile (called an I antigen); one is heat stable (called an S antigen). These occur together in a complex referred to as LS. In addition the nucleoprotein, characteristic of all viruses, acts as a separate antigen (NP antigen). None of these antigens appears to be related to neutralizing antibodies or antibodies which agglutinate the elementary bodies. The antigens responsible for neutralizing antibodies and agglutinins therefore may be other substances or possibly combinations of several chemical entities.<sup>16, 17</sup>

Studies on the resistance of viruses to various physical and chemical agents have yielded important information. In general animal viruses are killed in a few minutes by temperatures like that of pasteurization or even as low as  $56^{\circ}\text{C}$ . They are highly resistant to freezing and will remain alive at  $-76^{\circ}\text{C}$ . for a year or longer.<sup>18</sup> They are readily preserved by desiccation *in vacuo* after rapid freezing (*Lyophil*). Disinfectants like phenol, formaldehyde and iodine quickly inactivate them. Ultraviolet light is rapidly destructive. With few exceptions, sulfonamid drugs and penicillin are usually ineffective in treating virus infections. An example is seen in the experiments of Krueger, and others.<sup>18a</sup> Since these drugs appear to act through some effect on respiratory enzyme systems, it might be inferred that the respiratory systems of viruses, if any, are fundamentally different from those of many bacteria.

**Demonstration of Viruses.**—Since viruses are both ultramicroscopic in the ordinary microscopes and noncultivable in nonliving

material, it may appear strange that we have any information whatever concerning them. It must be admitted that our knowledge of the viruses is extremely limited. We know nothing of their metabolism, and only those that make themselves conspicuous by producing disease are recognized. If it were possible to make them induce changes (grow) in dead matter (artificial culture media) we could study them better. But, as far as is known, no such saprophytic properties of viruses have been observed. Viruses seem to be obligate *intracellular* parasites or pathogens, deriving their life only from living cells. What information we have, aside from the physical and chemical data, noted above, is derived chiefly from methods involving their ability to produce disease and immunity. Thus, we can determine the resistance of viruses to heat, light, disinfectants and other conditions and substances only by subjecting the material containing them to the desired situation, and then injecting them into some plant or animal which they can infect and seeing whether or not disease or immunity results.

An important point, and one that the student should not overlook, is that the injection of an active virus into a susceptible animal, *especially by an abnormal portal of entry*, does not always result in *visible* disease, but that very often in such cases the animal becomes immune and a second, so-called "test infection" is necessary in order to determine whether the first injection had any immunizing effect. With a few doubtful exceptions such as rabies and psittacosis, *as far as is known at present, only an active virus can produce immunity. This is an important fact to remember*, as it is fundamental in our consideration of active immunity in virus diseases.

**Are Viruses Alive?**—The question of the living nature of the ultramicroscopic viruses is an extremely interesting one.<sup>13, 14, 19, 20</sup> Here we have bodies not certainly visible, of such size that they can consist of only relatively few protein molecules, noncultivable, having no detectable metabolism yet capable of enormous and rapid multiplication, capable of existing in clear fluids in extremely high concentration yet often undemonstrable except by pathologic activity. Are we dealing with living structures or with minute non-living particles or with fluids? A fluid, after all, is merely a mass of particles of extremely small size. Could a fluid be alive? If viruses cannot be made to metabolize anything outside of living cells, how do we know that viruses actually exist as separate living, autonomous entities? Might it not be that certain cells, stimulated in a certain way, give off an enzyme-like substance

capable of stimulating other cells to give off the same sort of lethal substance? Yet this stimulus must always come from outside. It never starts spontaneously. Actually, there are no final answers to these questions at present available. All we can say is that some viruses may exist as living bodies, like elementary bodies; some as crystals which are not living as the word is ordinarily defined by biologists; and some as mere aggregations of a few molecules of protein. Their true nature or structure remains a mystery. In any case, if serum or sap from an infected animal or plant be passed through the finest filter, the filtrate will be free from any known bacteria yet capable of producing disease unless, in the meantime, subjected to heat or certain disinfectants or other unfavorable conditions.

The fact that immunity results when animals recover from virus infections<sup>21</sup> suggests that the viruses are antigenic, and therefore protein, in composition. Chemical data support this view. It has been intimated that viruses may be related to the extremely primitive, subvital bodies which might have preceded cellular organisms. The fact that we know little of the structure or metabolism of viruses is all that can be advanced in support of this suggestion. The fact that viruses are strict parasites on higher cells suggests that they may have developed only after the evolution of cellular life. Viruses causing diseases of man and animals are generally assumed to be alive.

*Parasitic Status of Viruses.*—A very interesting suggestion concerning the origin and nature of viruses has been advanced by Green<sup>22</sup> and elaborated by Laidlaw.<sup>14</sup> We have seen that bacteria, becoming parasitic, lose their rugged independence of living hosts and lose some of their metabolic or synthetic powers, and their resistance to the outer world, through long generations of life in a sheltered environment where much of their food is prepared for them. Such bacteria may derive from their host only simple substances such as constituents of saliva or feces or they may take certain nutrient materials from the blood, or very complicated substances like vitamins, depending on how high a degree of parasitism (or dependence on host) they have evolved. They do not as a rule, however, require to live *inside* the host cells. They are extracellular commensals or pathogens. Advancing a step further, we may imagine that certain bacteria have undergone such permanent changes in size and metabolism that they can actually enter the host cell and become an intracellular parasite, capable of living only there, perhaps at the expense of the proteins and



other essential constituents of the cell, and losing more and more of their own synthetic and other physiologic powers.\* As these powers are lost, through indolence and lack of exercise thereof, size diminishes until nothing remains but a bit of substance, probably protein in nature, ultramicroscopic in size, able to pass through the finest filters, capable only of reproducing its kind by division, and capable of life only if furnished with the cell-substances of some particular animal or plant to which it has become adapted. It would, "as it were, live a borrowed life, truly the supreme summit of parasitism."† Such a parasite would possess, as characteristic of itself, only the protein transmitting the specific properties of the virus. It would resemble the gene of the geneticists, independent and incarnate!

So viruses may be supposed to have originated through the development of parasitism to its ultimate perfection; an evolutionary process of a highly successful sort if we regard first-rate parasites as the goal of nature; an involutionary or degenerative trend if we regard the independent, self-supporting creature as the universal ideal. The latter, carried to its logical, nonparasitic extreme could be only of the autotrophic type.

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\* Indeed, just such visible but extremely minute, intracellular, bacterium- and virus-like parasites are well known and constitute an important group of organisms known as rickettsias, which are discussed in Chapter 45.

† Laidlaw, "Virus Diseases and Viruses," 1939. By permission of The Macmillan Company.

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## CHAPTER 43

## DISEASES CAUSED BY VIRUSES

**A NUMBER** of diseases of human beings are caused by ultramicroscopic viruses, among them measles, rabies, smallpox, yellow fever, dengue fever, anterior poliomyelitis (infantile paralysis), encephalitis lethargica (sleeping sickness due to inflammation of the brain), chickenpox, herpes simplex (fever blister), warts, common cold and influenza. Numerous diseases of farm animals are also caused by viruses; common ones are hog cholera, swine influenza, foot and mouth disease, chicken cancer and fowl plague. Several virus diseases of animals also affect man: equine encephalomyelitis,

rabies, psittacosis or "parrot fever." "Mosaic diseases" of tobacco and tomatoes, and certain wilts and blights of other plants are also virus diseases.

It is obviously impossible to discuss all of these viruses and the diseases they cause in this book. We may list some of the more important diseases and viruses and classify them in convenient, but still unsatisfactory, ways, following this with a discussion of some that illustrate interesting or important facts.

TABLE XIX  
CLASSIFICATION OF SOME ANIMAL VIRUSES

Group	Tissues Principally and Visibly Affected*	Diseases Caused	Mode of Transmission*
Dermotropic . . . .	Skin; mucous membranes of nose and mouth	Various pox-like diseases (smallpox, fowl pox) herpes, warts, measles	Close contact; probably sputum, fo mites.
Neurotropic . . . .	Nervous tissues.	Poliomyelitis  Rabies Various encephal- itides	Unknown. Flies? Feces? Bites of animals. Mosquitoes?
Pneumotropic . . . .	Respiratory tract	Influenza Psittacosis Pneumonitis	Nasal and oral dis- charges.
Viscerotropic . . . .	Various internal or- gans	Yellow fever Dengue Louping ill Rift Valley fever	Mosquitoes Mosquitoes Ticks Mosquitoes
Neoplastic . . . .	Various	Fowl sarcoma Fibroma of rabbits Myxoma of rabbits Fowl leukemia	Unknown Unknown Unknown Unknown

\* Under natural conditions.

**Classes of Viruses.**—A classification of the viruses of animal disease may be based on the types of tissues or organs principally affected. Another sort of classification could be based on mode of transmission. Such a list may be constructed as shown in Table XIX.

Any such classification, while convenient for purposes of discus- sion, is highly arbitrary and does not take into consideration the fact that, although certain tissues may be those visibly or prin- cipally affected, other tissues may also be involved so that another

grouping, which we might call *pantropic*, would include many of those classed above. For example, psittacosis becomes generalized and affects liver, spleen and gastro-intestinal tract, but this differs in different animals; smallpox can become generalized, and so on. One is at a loss to place some viruses, such as that of mumps and of fowl plague in the table. Further, the tissue affinities of some viruses can change completely.<sup>1, 1b</sup>

**Variations in Tropisms (Tissue Affinities) of Viruses.**—When viruses grow in the body they usually attack certain specific cells or organs. For example, the virus of yellow fever, when transmitted in nature by the bite of mosquitoes, affects principally, but not exclusively, the liver cells. It is spoken of as a *viscerotropic virus*.

If, however, the blood of a person or monkey suffering from yellow fever be injected artificially into the *brains* of mice, these animals develop *encephalitis* (inflammation of the brain) and die with *no evidence of damage to the liver*. If the brains of such mice be finely ground in fluid and injected into the brains of other mice, and these brains into still other mice and so on, through several brain-to-brain transfers, the virus appears to lose its ability to cause any damage to the liver or, indeed, *any visible effect* when injected *subcutaneously* into susceptible monkeys. The virus is said to have lost its affinity for the viscera (liver, etc.) or its *viscerotropic* property. If, however, such mouse brains are injected into the *brain* of a monkey or almost any other animal, especially rodents,<sup>1, 1a</sup> the brain virus nearly always produces a *fatal* encephalitis just as it does in mice and is confined largely to the nervous system, having little or no effect on the liver. It is said to have become *neurotropic*. Influenza virus can be shown to adapt itself to various tissues of chicken embryos in the laboratory, with alterations of virulence. Other viruses may acquire affinities for different organs or tissues naturally. It is evident that viruses undergo variation suggestive of some phases of bacterial variation.<sup>1b, 1c</sup>

**Intracellular Inclusions in Virus Diseases.**—When viruses invade tissue cells, they often produce changes in the cells which can be recognized with the microscope. Certain granules, or groups of granules, develop *inside* the cells. These are often quite characteristic in appearance and staining properties and may enable a skilled pathologist to diagnose the disease from the appearance of these granules or bodies. Since the bodies are included in the cells they are spoken of as *intracellular inclusions*

Bacterial infections do not regularly cause such definite inclusions to form. Some of these inclusion bodies are typically present only in the cytoplasm and are called *cytoplasmic inclusions* (Fig. 359). Others are found only inside the nucleus and are called *intranuclear inclusions* (Fig. 360).

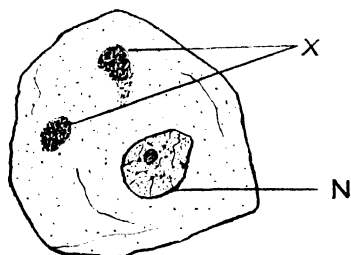


Fig. 359.—One type of cytoplasmic inclusion bodies shown at *X*. Nucleus at *N*.

The nature of these inclusions is obscure. Some workers regard them as actual, intracellular colonies of the virus particles. Others believe them to be merely changed cellular material or detritus (remains of broken down tissue), representing damage by the virus but not the virus itself. Probably in some cases, as in lymphogranuloma, psittacosis and related diseases, the granules actually are virus particles. In other instances, as yellow fever, the question

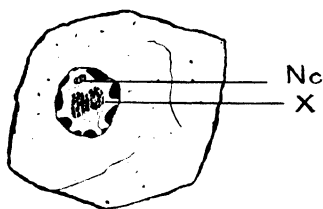


Fig. 360.—Intranuclear inclusion bodies in yellow fever. *Nc* Nucleolus; *X* inclusion bodies. Note the peculiar lobulations at the nucleus wall. (Redrawn from Cowdry and Kitchen.)

must be left unanswered. Yellow fever inclusion bodies are of two types: intranuclear<sup>2</sup> and intracytoplasmic.<sup>3</sup> The cytoplasmic bodies are doubtless cellular detritus (Fig. 360).

**Rivers' Postulates in Virus Diseases.**—Viruses were unknown at the time of Koch, so that he failed to take these invisible, non-cultivable agents of disease into consideration in stating the criteria by which the causal relationship of a pathogen to a disease might be determined. Rivers, in 1937,<sup>4</sup> outlined criteria similar to

Koch's postulates, which might apply in the case of viruses. Essentially these are as follows:

1. The virus must be present in the host cells showing the specific lesions, at the time of the disease, or in the blood or other body fluids.
2. Filtrates of the infectious material (blood, etc., or tissue triturates) shown not to contain bacteria or other visible or cultivable organisms, must produce the disease or its counterpart in appropriate animals or plants.
3. Similar filtrates from such animals or plants must transmit the disease.

Immunological and histological studies should confirm the specificity and identity of the diseases caused, but this is not always possible. Some of the many great difficulties in such demonstrations lie in the fact that (1) susceptible animals are not always known or available; (2) many animals have latent virus infections of their own which become active in laboratory experiments and may produce lesions and symptoms closely simulating those of the virus under investigation;<sup>5</sup> (3) latent viruses may become mixed with the virus injected;<sup>6</sup> (4) immunological cross reactions may occur, or the virus injected may cause little immunity while a latent virus may stir up its own immunity; (5) in some instances a virus requires the synergistic aid of a bacterium or another virus to cause typical disease.<sup>7, 34</sup>

**Some Virus Diseases.**—In dealing with bacterial diseases we find each to present problems peculiar to that infection yet to exhibit characters illustrative of certain phenomena common to many bacterial infections. So, also, we encounter a great variety of problems in virus diseases, related to portals of entry, modes of transmission, insect vectors, immunity and the like, many of which are common to bacterial phenomena. We should give some attention to these at this point.

One of the virus diseases of man and animals which has been most intensively investigated is yellow fever. The facts concerning yellow fever and yellow fever virus serve to illustrate numerous phenomena which have been discovered in connection with studies of other viruses. In addition, the history of the development of our knowledge of yellow fever is of some interest and we may, therefore, consider first this disease and its causative agent in some detail.

**Yellow Fever.**—Yellow fever is endemic only in certain tropical regions. The disease was probably brought from Africa to the

western hemisphere by Portuguese and Spanish explorers and slave traders as early as the year 1500. It was later distributed through all the Caribbean Islands and Central and South America, taking a terrific toll of life wherever it appeared. For centuries the "yellow jack," as it was called, made life in the tropics a matter of extreme peril. No one knew how it spread or how to avoid it. Many curious notions about it sprang up. In 1558 Rochefort stated that, "The air of all those islands is very temperate and healthy when one is accustomed to it. . . . But some years since, the islands were afflicted with malignant fevers which the physicians considered contagious. The bad air was brought by some ships which came from the coast of Africa." This notion that "bad air" was a cause of disease was quite understandable and was an ancient one. The term *malaria* means "bad air." El Hastio da Rocha Pitti in 1720 said, "In the year 1686 commenced in Pernambuco [Brazil] that terrible plague which must be attributed to the sins of the population of these provinces, corrupted by the vices into which they were enticed by the wealth and freedom of Brazil." Pezuela in 1648 said, ". . . in the capital (the epidemic) continued to carry away its victims without regard to rivalries and passions."<sup>8</sup> Hindle states, "In the 90's of the last century the crews of ships going to South and Central American ports were to a large extent shanghaied [kidnapped and forced into service] and this captain himself obtained command at the early age of twenty-one by volunteering to take the ship to Santos. On this particular voyage all except three of a crew of twenty-one died of yellow fever, so their fears were fully realized."<sup>9</sup> When the French tried to build a canal through Panama, the laborers died in such numbers from yellow fever, malaria and dysentery, that the attempt was abandoned. During the Spanish-American War, many hundreds of American soldiers died of the disease in Cuba, even after a general campaign of "sanitation" had been carried out.

The military authorities finally appointed a yellow fever commission composed of James Carroll, Jesse W. Lazear and Aristides Agramonte under the direction of Walter Reed (Fig. 361). These men, armed only with the very little evidence then available, set out with a grim determination to solve the problem of yellow fever.

Many different kinds of bacteria had been blamed as the cause of yellow fever. Reed first made a thorough bacteriological investigation and came to the conclusion that the disease was not due to bacterial infection.

A generally held idea was that fomites (bedding, clothing,

dishes, etc.) contaminated with the vomitus or feces of victims of yellow fever were very dangerous vectors of the disease-producing agent, whatever it might be. Indeed, some time before, a ship in New York harbor had had some yellow fever patients on board. A shirt cast up days later by the waves on Long Island, some miles away, was seen by a gentleman strolling on the sand. He poked at the shirt with his cane but did not touch it. Some days afterward yellow fever broke out in the community and the gentleman with the cane, being one of the first to succumb, was thought to have contracted the disease from the shirt and spread it to his neighbors.



Fig. 361.—Walter Reed.

Another idea, advanced by Carlos Finlay (Fig. 362), a Cuban scientist of Scottish descent, was that yellow fever was transmitted by the bite of *Aedes aegypti* mosquitoes. Finlay's idea was treated rather contemptuously by many wise men of the times. It seemed preposterous!

Walter Reed and his colleagues arranged experiments to test these two hypotheses. They built a little screened house, divided into two halves. They put beds, chairs and other simple furnishings on each side. In one compartment they piled blankets, sheets, bedding, clothes, and other fomites of men who had died of yellow



fever. The things were filthy with dried vomitus and feces. No mosquitoes were allowed in this compartment, but a number of the insects, known to have bitten yellow fever patients, were placed in the other compartment which was clean and free from fomites.

Since Reed and his co-workers had not found any animal which was susceptible to the disease, and which they could use for the experiment, human volunteers were asked to live in the compartments for days; to be imprisoned with lurking, flying death; to sleep in the beds; live in the clothes and eat from the dishes soiled by men who had died of yellow fever. It was not a pleasant invitation but there were numerous volunteers among the American troops! For days nothing happened. Then, one after another, men in the mos-



Fig. 362.—Carlos J. Finlay.



Fig. 363.—William H. Welch. (From the painting by Martin Frobisher, Sr. Photograph by the courtesy of Dr. W. G. MacCallum.)

quito compartment became ill with yellow fever. Fortunately none of the men died. The men in the other compartment remained well! The secret was at last wrenched from nature! The mysterious messenger of death was at last revealed as a certain mosquito (*Aedes aegypti*). Meanwhile Lazear, allowing himself to be bitten in a yellow fever hospital by an infected mosquito, lost his own life. Carlos Finlay's "ridiculous ideas" were fully verified.<sup>10</sup>

Although the *means* by which yellow fever was transmitted was

known, the actual *cause* of the disease was not. Extensive bacteriological investigations had failed to reveal any organism which seemed to be definitely associated with the disease. Walter Reed, at the suggestion of that great physician and founder of medical science in America, William Henry Welch (Fig. 363), tested the filterability of the infectious agent in the blood of yellow fever patients and easily proved that the causative agent was not an ordinary bacterium but an *invisible, noncultivable, filter-passing* virus. Thus, not only was the transmitting insect but the nature of the causative agent revealed.<sup>11</sup>

The remainder of the story is one of confirmation of Reed's work, study of the virus, and campaigns against *Aedes* mosquitoes. Yellow fever disappeared from Cuba! The Panama Canal became a practical project. The islands of the Caribbean became more healthy, and the "yellow jack" retreated, snarling, as it were, to South America. In 1932 Soper, Cardoso, Seraphim, Frobisher and Pinheiro made the discovery that yellow fever persists in remote parts of the wooded and rural portions of the continent, where it is transmitted by biting insects other than *Aedes aegypti* and probably is maintained among wild animals as well as human beings. It is referred to as "jungle yellow fever."<sup>12, 13</sup> Since Reed's time intensive study of yellow fever has continued.

Two of the most important discoveries were the susceptibility of *rhesus* monkeys<sup>14</sup> and of white mice<sup>15</sup> to the disease. These findings greatly facilitated research, as no means of maintaining viruses in tissue cultures was then known and large scale human experiments were impracticable. Complement-fixing antibodies were first demonstrated in yellow fever by Frobisher<sup>16</sup> and have since been found in other virus diseases.<sup>17</sup>

**Immunization Against Yellow Fever.**—When yellow fever virus, modified by mouse-brain passage so as to become *neurotropic*, as described above, is injected into the human body by any route *other than the nervous system*, no visible attack of yellow fever occurs but the virus stimulates the production of very potent antibodies and the person is thenceforth immune to yellow fever.

A very curious phenomenon related to this variation in tropism was observed after it was found possible to cultivate the yellow fever virus in chick-embryo Tyrode-solution medium. It was found that neurotropic virus completely lost its neurotropic virulence if the *nervous tissues* (brain and spinal cord) of the chick embryo were removed before mincing their tissues for the medium! The virulence of the virus was so reduced in such cultures that it

seemed incapable of producing either the classical viscerotropic or the neurotropic form of the disease. It was spoken of as *pantropic*. It did, however, produce immunity, and it has been used to immunize millions of persons, especially in Brazil and the U. S. armed forces.<sup>19</sup> It was later found that this loss of virulence could occur as a result of natural variation.<sup>18</sup> The phenomenon offers an excellent field for research.

The present method of preparation of yellow fever vaccine consists in inoculating a virus of reduced virulence into chick embryos in the shell. The inoculum for the embryos consists of fluid from a tissue culture of measured, attenuated virulence. (See section on methods of cultivation, page 162.) Further steps include incubating for about four days and then removing the embryos and grinding them very finely in a suitable suspending fluid. Millions of American military personnel have been protected from yellow fever by this vaccine which is highly satisfactory. Certain initial difficulties with jaundice due to the inclusion of a certain icterogenic factor in human serum in the suspending fluid were overcome by discontinuing the use of the serum.<sup>19a</sup>

**Poliomyelitis.**—Infantile paralysis or, more properly, anterior poliomyelitis, is an acute febrile disease, world-wide in distribution, most common in children as a rule but not confined to childhood and has a true virus as causative agent. It is characterized, as are many virus diseases, by sudden onset and a febrile attack with nausea, headache, sometimes stiff neck, and muscular pains. There is evidence that in many instances the disease is short and mild, often passing unrecognized, with complete recovery and presumably life-long immunity. In a relatively small percentage of cases the disease is severe, and myelitis (muscular disease) with paralysis and degeneration occurs. There is extensive damage to nervous tissues, including those which are in association with the muscles, as well as to the walls of blood vessels.

Studies of the virus show it to be one of the smallest ( $10\text{--}15\mu$ ). It is possible that it may exist as liquid crystals somewhat like plant viruses.<sup>20</sup> There appear to be several strains, with somewhat different geographical distributions, clinical peculiarities and immunological differences. Laboratory investigation of poliomyelitis has been hampered by the fact that monkeys were for a long time the only known susceptible experimental animals. A strain of poliomyelitis virus now referred to as the Lansing or mouse-adapted strain was isolated in monkeys in 1937 by Armstrong from a fatal human case. By passage of the virus through eastern cotton

rats and later through white mice, it became adapted to mice so that it could be used for experimental purposes, such as tests of the neutralizing power of patients' and convalescents' sera. Only one or two poliomyelitis viruses have undergone adaptation to mice.

Serological studies in either mice or monkeys with any of the strains of virus have been disappointing in some respects. For example, there seems to be little correlation between the Lansing virus-neutralizing properties of human serum and either the development of poliomyelitis or recovery from it.<sup>21</sup> That is, persons may develop poliomyelitis whether or not they have neutralizing antibodies, and they may or may not develop neutralizing antibodies as a result of having the disease. Finally, as stated by Turner and Young, "The neutralization test with the Lansing strain is of no value as a diagnostic procedure in doubtful clinical cases."<sup>21</sup> Tissue immunity may be of greater importance in poliomyelitis than is humoral immunity. This question is more fully discussed by Howe and Bodian.<sup>22</sup> Complement fixation tests, useful in yellow fever, lymphogranuloma venereum and a number of other virus diseases, are not available in poliomyelitis.

The distribution of poliomyelitis virus, as indicated by tests for neutralizing antibodies against the Lansing or one of several other strains, with sera collected among various populations, seems to be fairly general and widespread although only occasionally does a severe paralytic or fatal case occur.<sup>23</sup> The means of transmission is not clear. The virus is found in the human intestinal tract<sup>24, 24a</sup> and has repeatedly been found in feces of patients and of convalescent carriers and normal persons. It also occurs in sputum, sewage, and house flies<sup>25</sup> from privies containing infectious feces and therefore probably finds many pathways open for transfer from one person to another. Cases seem to develop particularly during summer and fall, and more commonly in suburban and rural areas than in cities. There are no generally accepted means of prophylaxis. Complete avoidance of sputum, sewage and house flies might or might not give protection, provided it were possible so to isolate oneself.

**Psittacosis (Parrot Fever).**—Psittacosis and its virus illustrate several points in common, and also in contrast, with yellow fever, and it will be of interest to discuss some of them briefly. The fact that human beings can contract a form of pneumonia from sick parrots was recognized by Ritter as early as 1880. The disease is a dangerous one. Various bacteria were designated as the cause of the disease, notably, a gram-negative rod once classified as *Sal-*

*monella psittacosis* but now regarded as identical with *S. typhimurium*. The widespread occurrence of "parrot fever," or psittacosis, in 1929-1930 stimulated research and, as in yellow fever, it was found that the causative agent is not a bacterium. It is present in the blood and sputum of human patients as well as in tissues of man and birds and will pass through Berkefeld filters.<sup>26</sup>

*Large elementary bodies* are found in and about tissue cells of organs (spleens, livers) of infected parrots, mice, etc. These appear to have a complex structure and may contain more than one antigenic substance. An analogy is found in the studies of Craigie and Wishart,<sup>27</sup> Rivers,<sup>28</sup> Parker<sup>29</sup> and others, who have demonstrated antigenic complexity in smallpox bodies. The bodies of psittacosis sometimes are bacillary in form.

This virus, like other viruses, will not grow except in contact with living tissue cells. It may be cultivated in tissue cultures and chick embryos. On the chorio-allantoic membrane pock-like lesions are described. Thus we see that the virus of psittacosis partakes of some of the properties of both yellow fever and smallpox viruses, but shows differences from each which illustrate the danger of making generalized statements about any virus.

Psittacosis is *transmitted* by dried nasal secretions and feces of parrots and such infected material may be blown or otherwise transmitted for considerable distances from the infected birds.<sup>30</sup> Many avian species, other than parrots, etc., are also susceptible. Canaries, finches, chicks and others may transmit the disease to each other and to man.<sup>31</sup> Similar, probably identical diseases in non-psittacine birds are referred to as *ornithosis*. Birds often become infective carriers. The disease was endemic in commercial aviaries engaged in raising parrots and related birds for pet shops.<sup>31</sup> It is also found in pigeons.<sup>32</sup>

In man, if the disease is contracted naturally, *i.e.*, by inhalation of dust infected by birds, the disease takes the form of a pneumonia, the virus being demonstrable in the sputum and blood. This is used for diagnosis by injection into mice. Transmission from man to man is uncommon.

When injected into the brains of mice, rabbits, guinea pigs or monkeys, the virus produces a meningo-encephalitis (inflammation of brain and covering membranes) but does not affect the viscera. No marked change in tropism, such as is observed in yellow fever virus, however, has so far been described for psittacosis virus, although there is no reason to suspect that such a variation might not occur.

**Immunity.**—Man and animals, after recovery, are somewhat refractory to reinfection, but immunity to psittacosis is not so marked, prolonged or readily demonstrable as in yellow fever or smallpox. The presence of immune bodies in the blood of animals or men recovered from the disease is demonstrated by mixing their serum with infectious material (ground-up spleen, etc.) and injecting it into mice. The mice are, in great part, protected. This is called a *neutralization test*. A similar test is used in the demonstration of yellow fever antibodies, as well as antibodies against poliomyelitis and a number of other virus diseases.

As is true of rabies vaccination, possibly a certain degree of immunity to psittacosis may result from the injection of virus inactivated by formalin.<sup>32</sup> Apparently when infection occurs later, psittacosis virus can still multiply in animals so immunized, but is held to a very low level. Continued immunity appears to depend on premunition (continuous presence of active virus in the body).

**Abnormal Portal of Entry.**—Psittacosis virus, injected into monkeys or human beings *intramuscularly* (as contrasted with intracranial and respiratory routes of infection), produces only a mild illness which results in a relatively refractory state. This fact has been made use of in the protection of human beings engaged in laboratory investigations of the disease. It is evident that the portal of entry plays an all-important role in psittacosis as well as in yellow fever. Similarly, in rabies, Webster developed a virus, by passage through mouse brains, which produces classical rabies when injected *intramuscularly* or *intracerebrally*. If introduced by the *intraperitoneal* route, however, susceptible mice remain healthy, even with 10,000 lethal doses, and become immune!<sup>33</sup>

The processes of immunization with vaccinia virus, yellow fever virus and psittacosis or rabies virus represent three different principles: (1) Vaccinia virus is human smallpox virus *attenuated by passage through other animals* (cows). (2) Yellow fever virus depends on a reduction of virulence which is *artificially* induced (tissue culture) but which also probably occurs in nature. (3) Psittacosis virus or the form of rabies virus mentioned above is not attenuated or changed in any way, but is introduced into the body by an *unnatural portal* (intramuscularly for the former and intraperitoneally for the latter), and fails to produce a serious disease but acts as an antigen nevertheless.

**Bacterial Infections in Virus Diseases.**—Animals or persons infected with some viruses seem to be especially susceptible to simultaneous infection with certain bacteria. This secondary infection

often produces severe or fatal results in virus diseases which, in the absence of the bacteria, are in themselves relatively mild. Colds or influenza, for example, may make the tissues unable to resist infection with pneumococci or streptococci. A good example of this combined action is seen in hog cholera or swine fever and, as shown by Shope, in swine influenza.

**Hog Cholera.**—This disease is caused by a filterable virus which occurs in the blood, urine and other fluids and in the organs and feces of infected animals and which can be demonstrated in filtrates of these by inoculation—a good example of a pantropic virus.<sup>34</sup> The virus dies out in a week or so in soil. It is not as resistant to drying as is psittacosis virus. Animals sometimes recover and are immune for a long time. Good immunity usually results from the simultaneous injection of active virus (in blood) and immune serum. Occasionally the virus or the serum fails to act properly. The hogs may then die of immediate infection or later contract the disease.

In some epizootics\* of the disease, which is transmitted by infected soil, water, food, etc., an organism of the *Salmonella* group (*Salmonella choleraesuis*) accompanies the virus, causing great mortality, with severe intestinal symptoms. Likewise, an organism of the *Pasteurella* group (*Pasteurella suis*) may accompany the virus (with or without *S. choleraesuis*) also causing a very severe epizootic with pulmonary involvement. The association of these bacteria with the disease formerly caused great confusion. For a long time, as a result of the investigations of Salmon and Smith in 1885, *S. choleraesuis* was regarded as the cause of swine fever. De Schweinitz and Dorset, however, in 1903, following Walter Reed's discovery of the virus of yellow fever, proved the virus nature of the disease and showed that the bacteria, although frequently present, were only secondary invaders after the virus.

**Swine influenza**, some forms of which are colloquially called "thumps," was shown by Shope to be due to a filterable virus related to that of human influenza. The disease is usually very severe when occurring under natural conditions, yet Shope, using his filtered and bacteria-free virus, could produce only a very mild disease with it. He later found that the natural epizootics are severe because, in such instances, there is associated with the virus a gram-negative bacillus closely resembling *Hemophilus influenzae* and which he called *H. influenzae (suis)*. This is present in most

\* Unusual prevalence of a specific infectious disease among animals.

swine. Instillation of the bacilli alone into the hogs caused no disease whatever, a classical example of microbial synergism. The disease is transmitted by nasal secretions.<sup>35</sup>

*Immunological Aspects of Swine Influenza.*—Some aspects of immunity in swine influenza are of particular interest.<sup>36</sup> As in human influenza, repeated attacks are common. Prophylactic injection with the virus alone produces a moderately good immunity. Prophylactic injection with *H. influenzae (suis)* causes a reduction in the severity of the combined infection but no immunity to the virus.

If human influenza virus alone be used for swine immunization, no effective immunity to swine influenza results. But if human virus and the bacterium of swine influenza are used together, the swine are immune to swine influenza. Animals recovered from infection with swine virus are immune to human virus.<sup>37</sup>

*"Hibernation" of Swine Influenza Virus.*—This disease occurs in yearly or seasonal epidemics eight or nine months or more apart. There had not, hitherto, been a satisfactory explanation of how the virus survives during the intervals. It was found by Shope in 1939 that the virus can survive in larvae of the pig lungworm (a common parasite of domestic pigs), remaining latent there through the whole developmental cycle of the parasite, which may last for months or years. The virus passes from pig to pig with the lungworms *via* earthworms (the earthworm is one of the hosts in the development of the immature lungworms). Once gaining entrance, with a lungworm, to the lungs of a new pig, the virus only awaits the liberating or activating effect of some agent such as infection with *Hemophilus influenza (suis)* or some unknown chemical or mechanical irritation. The implications of this discovery in relation to many other diseases are very interesting.<sup>38</sup>

**Human Influenza and Colds.**—The symptoms of these diseases are not entirely characteristic and may be present in cases of either colds or influenza. The general conception of a cold, however, is a disease confined largely to the nose and upper respiratory tract, with edema of the nasal mucosa and mucous discharge, not particularly disabling and sometimes accompanied by a slight sore throat or cough. Influenza, on the other hand, is generally described as a febrile disease, extremely weakening, accompanied by muscular pains, sweating, and sometimes by the symptoms of the common cold as well. In view of the extreme variability of the symptoms in both diseases and their tendency to overlap, it is a question whether one is justified in making a distinction. It is



possible that the two conditions may represent different reactions on the part of the host to the same infectious agent, or, on the other hand, that the host may react similarly to different infectious agents.

*Colds* have been shown to be due to a filterable virus. The results of various transmission experiments with monkeys and human volunteers might be interpreted as indicating that "grippe," influenza and common colds are identical since the diseases are so difficult to distinguish clinically and because virus taken from what are, clinically, cases of influenza may produce what seem to be "colds" in volunteers. However, the common cold is probably due to one distinct virus, or group of related viruses, and influenza to another. One may be a "variant" of the other. Dochez and his coworkers have cultivated cold virus in tissue cultures and found it different in many respects from influenza virus. For example, the virus of colds appears to be anaerobic.<sup>39, 40</sup>

The common cold, uncomplicated by any virulent, infectious bacteria, is usually a relatively mild disease. However, certain bacteria, particularly beta-type hemolytic streptococci, influenza bacilli and pneumococci, are particularly apt to gain a foothold in the inflamed tissues of the nose and throat, and the combined or subsequent bacterial infection not infrequently results in serious disease.

*The virus of epidemic influenza* has been extensively studied by Laidlaw, Andrewes and Smith<sup>41</sup> in England and by Chapman and Hyde,<sup>42</sup> Francis, Horsfall,<sup>43</sup> Lennette and many others in this country.<sup>44</sup> The virus in nasal and oral secretions is capable of passing filters and of infecting man, ferrets and mice and possibly swine *when instilled intranasally*. It does not infect, but may immunize, when injected intraperitoneally or subcutaneously (immunization by unnatural portal of entry).

The virus is one of the smaller group, round or ovoid in form. Its physical and chemical properties have been carefully determined.<sup>51</sup>

A curious agglutination of chick erythrocytes in the presence of influenza virus was discovered by Hirst.<sup>45</sup> Virus cultivated in chick embryos has a definite agglutinating effect. The reaction is prevented by immune serum. Hirst developed a scheme for determining the potency of anti-influenza serum by titrating the serum in the presence of the red cells.<sup>46</sup> That this observation is probably of very fundamental significance is indicated by the fact that a similar method has been adapted to vaccine virus studies.<sup>47</sup>

**Types of Human Influenza.**—It is essential to differentiate between the several types of human influenza. First, there is *endemic* influenza. Clinically this is indistinguishable from the *epidemic* disease. Endemic diseases are those which are always present in constant numbers in the population, cases or small groups of cases occurring sporadically here and there, now and then. It may be that endemic influenza is one or more infectious diseases, but the cause (or causes) is not known. These sporadic cases, although diagnosed clinically as “influenza,” may in reality represent similar host reactions to a variety of infectious agents not yet identified.

*Epidemic influenza*, on the contrary, has a more distinct yet not unique etiology. At least two types of epidemic influenza virus have been discovered. The older one, discovered by Smith, Andrews and Laidlaw in 1933,<sup>48</sup> now called influenza virus A, seems to be most widely distributed. Influenza virus B was differentiated in 1940 by Francis<sup>49</sup> and by Magill.<sup>50</sup> Possibly a third type may be demonstrated. An interesting comparison of viruses A and B was reported in 1943.<sup>51</sup> The A and B viruses, while causing clinically indistinguishable diseases, and although very much alike, are entirely distinct in an immunological sense.

The type of influenza which occurred in highly lethal form in the 1918–1919 world-wide epidemic may have been due to still another form of the virus, which is sometimes differentiated as “the virus of pandemic influenza.” The true etiology of the 1918–1919 epidemic is still a matter of surmise.

The influenza viruses are cultivable in chick-embryo-Tyrode medium and on the chorioallantoic membranes of chick embryos, and may be used for vaccine. Promising results with combined formalized vaccines A and B were reported in 1944 by the Commission on Influenza, of the U. S. Army Medical Corps.<sup>52</sup>

Artificial infections produced by inhalation of type B virus appear to reduce the severity of infections with the same virus acquired four months later, but do not prevent them. Possibly greater resistance to infection would be found after intervals less than four months.<sup>52a</sup>

Another promising sort of influenza vaccine has been developed by Friedewald.<sup>52b</sup> The virus is adsorbed on the surface of particles such as dead bacteria in paraffin oil and in this particulate state seems to be more effective antigenically. Human immunization experiments have not as yet been tried. The basic principle (enlargement of the virus antigen masses by adsorption on definite particles) may be generally applicable.

**Lymphogranuloma Venereum.**—This disease, probably world-wide in distribution, though more common in tropical regions and in the Negro race, is due to a virus having certain properties in common with that of psittacosis. The disease is transmitted chiefly by sexual contact, rarely by other means, and is most prevalent in sexually promiscuous persons during the ages of greatest sexual activity. The virus has its portal of entry through various mucous membranes, especially those of the genitalia and anorectal region. It may pass through the skin, via abrasions and cuts, or through the respiratory tract. It is usually not so fatal as syphilis and often heals spontaneously. However, it is capable of doing great damage.

It begins as a small vesicle or ulcer on the genitalia. This may heal and pass almost unnoticed by the patient. Later, nearby lymphatic glands become swollen and inflamed, forming buboes and discharging much pus containing the infective agent. This process may become very extensive. As a result of blockage of the lymph channels, huge swellings (elephantiasis) occur in the surrounding parts and legs. Extensive and destructive chronic ulcerations result, especially in the female, and involve the vulvo-anorectal region. Anorectal involvement also occurs in males. Scar formation may cause serious impairment of intestinal function and other difficulties. The virus may persist in apparently cured persons for weeks or months, perhaps years.<sup>53</sup>

The virus itself is one of a group of several, including psittacosis, ornithosis, vaccinia, inclusion conjunctivitis and meningo-pneumonitis,<sup>54, 55, 55a</sup> which are characterized by elementary bodies of relatively large size ( $125\mu\mu$  to  $250\mu\mu$ ) which can be stained with aniline dyes (gram-negative), studied with ordinary microscopes, and many of which seem to undergo some sort of cyclical morphological changes as they develop in the cells of infected animals.<sup>55b</sup> These morphological changes in lymphogranuloma virus involve multiplication of the tiny elementary bodies or initial bodies into chains and masses (plaques), the masses finally enclosing themselves in little vacuoles or vesicles in the infected cells and tissues. The vesicles and masses of granules increase in size and finally rupture, liberating many elementary bodies in the surrounding tissues (Fig. 364). A modified cycle with more extensive development of elementary bodies is said to occur in tissues already infected. Special staining properties of the infective elements, with Giemsa's stain, in various stages of development, are described as characteristic for the different stages of the life cycle. A very similar

developmental process has been seen in the psittacosis virus. The vaccinia cycle is not so clear. Similar structures and cyclical changes are seen in the other viruses in the lymphogranuloma-psittacosis group, but whether they represent the same processes and have the same significance in all is not clear.

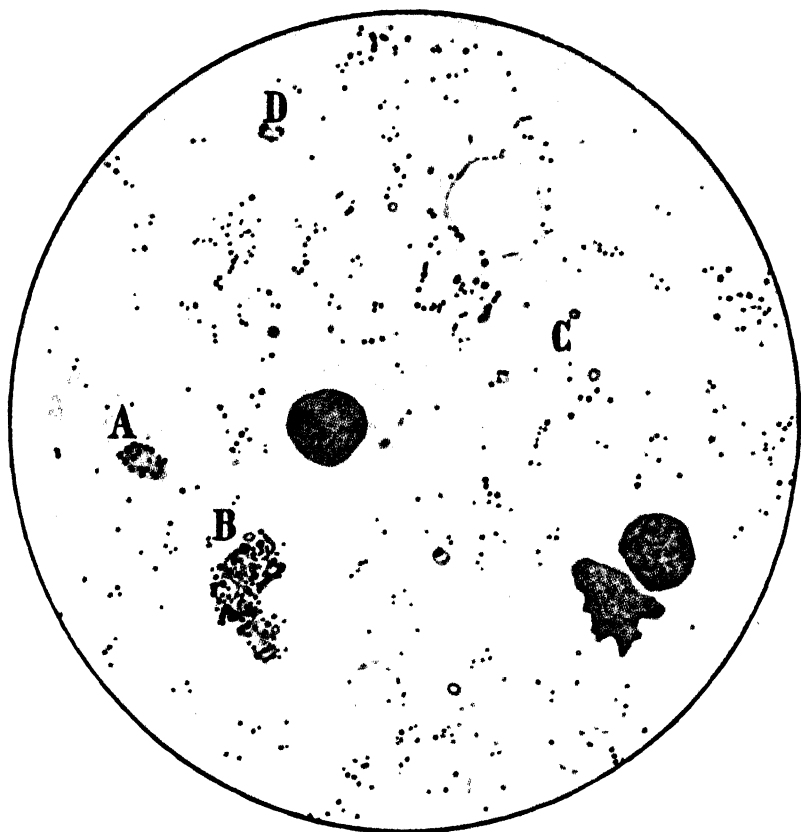


Fig. 364.—Yolk sac smear showing elementary bodies of lymphogranuloma venereum virus in various stages of development. *A* and *D*, Groups of initial bodies. *B*, Elementary bodies being liberated by fragmentation of a mass. *C*, Large elementary bodies containing a vacuole. (Courtesy of E. R. Squibb & Sons, New York.)

A significant observation in regard to the lymphogranuloma and related pathogenic agents is that they form toxins similar to bacterial endotoxins against which specific antitoxic sera may be produced.<sup>55c</sup> This has not been demonstrated in the case of any of the

true viruses previously discussed, or any of the rickettsiae to be described in a later chapter.

The virus of lymphogranuloma venereum, as well as that of psittacosis and of meningo-pneumonitis, is cultivable in chick embryos, multiplying especially well in the yolk sac.<sup>56</sup> They may also be developed in the brains of experimentally injected mice or in the pus from draining buboes. This makes it possible to obtain large amounts of the elementary bodies for use in research and diagnostic tests. One diagnostic test consists in the intradermal injection of heat- or phenol-killed suspension of the elementary bodies. In persons who have been infected for about two weeks or more there develops, after forty-eight to seventy-two hours, a characteristic papule with swelling and redness around the site of the injection. This is known as a Frei reaction, having been discovered by Frei in 1925. Complement fixation tests with the egg antigen are of value in studying the incidence and distribution of lymphogranuloma, and routine testing of sera submitted in compliance with marriage laws for syphilis control in some states might be of assistance in revealing, and controlling the spread of, this venereal disease. No method of active immunization is available at present. Control at present centers around finding and treating cases and preventing the spread of the disease to others through sexual or other personal contact (such as sleeping in the same bed) or through clothing, towels, toilet articles, etc. Repression of prostitution and public education are always indicated in attempts to prevent the spread of venereal disease.

**Neoplastic Diseases.**—One of the great mysteries of human pathology is the etiology and nature of "cancer." There is a great variety of cancers or, more properly, tumors (swellings) or neoplasms (*neo* = new; *plasm* = tissue) of both plants and animals, and there is a variety of causative agents. Whether any of these is a living organism is still unknown with regard to human neoplasms, but several animal tumors are definitely known to be due to infections with viruses, while some plant tumors are due to bacteria (see section on plant diseases, page 472) as well as to viruses. Whether, by studies of the animal tumors, the secret of human "cancer" may eventually be solved, no one can say. There are many suggestive analogies between the infectious animal tumors and human neoplasms, but the actual relationship, if any, is still obscure.<sup>57</sup> Space does not permit us to discuss in detail this fascinating problem, but a few suggestive facts may be mentioned.

*Myxomatosis of rabbits* is a highly infectious, rapidly progressive

and almost invariably fatal disease of *domestic* rabbits. The virus is spread by discharges from eyes and nose and from the malignant, cancer-like, slimy tumors that occur all over the body. The virus spreads especially to the eyelids, internal organs, ears, and any areas in the skin where it may find entrance, as by abrasions, or injection. The tumors contain large amounts of the virus, and result from a cancer-like proliferation of the tissue cells. The tumors (or the virus particles) spread and metastasize (or establish new tumors) much as do malignant human cancers (Fig. 365).<sup>58</sup> The disease is not infectious for human beings.

*Fibroma of rabbits* is a disease especially of *wild*, cottontail rabbits, which bears a very remarkable relationship to the myxoma (slime-tumor) described above.

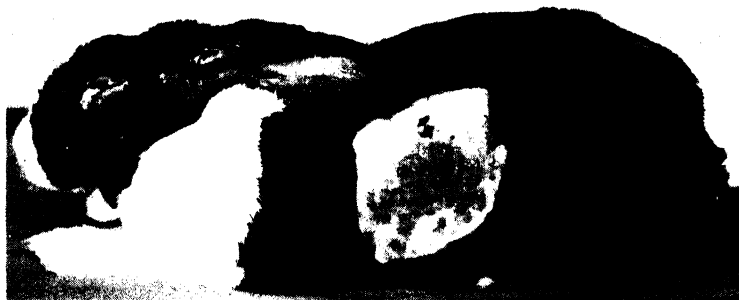


Fig. 365.—Myxoma of rabbit, showing soggy tumors spreading from site of inoculation in flank, and on ears, eyes and nose. (After Hyde.)

Fibroma virus causes large, hard, warty tumors in cottontails (Fig. 366). These tumors have a closer superficial resemblance to benign human tumors (which grow slowly and show little tendency to spread) than they do to malignant human cancer or infectious myxoma of rabbits. Fibroma disease is not usually fatal.<sup>59</sup>

One of the relationships between the fibroma and myxoma viruses that is of especial interest is the power of a few drops of fibroma-tissue juice (containing the virus) to *immunize completely against the myxoma virus* if instilled into the eyes or injected into the bodies of susceptible domestic rabbits a few days before the introduction of the myxoma virus. Likewise, cottontail rabbits *infected with myxoma virus* develop only mild, fibroma-like lesions. Cottontails so infected are immune to the natural fibroma virus. More interesting still is the observation that myxoma virus, maintained

for some time in the cottontail, produces lesions, when returned to the domestic rabbit, which are at first more like fibroma than myxoma. Most interesting of all is the demonstration by Berry,<sup>60</sup> Hyde,<sup>61</sup> and others, that fibroma virus will acquire all of the properties of the myxoma virus when treated with heat-inactivated myxoma material.

No explanation of these phenomena is offered. One may wonder whether fibroma virus is a "variant" or modified form of myxoma virus adapted to wild rabbits and, if so, how it lost its great killing power. Probably it is more logical to regard myxoma virus as a modified fibroma virus since the wild rabbit must have been the forerunner of the domesticated species. The process of change would



Fig. 366.—Cottontail rabbit with naturally occurring virus papillomas. One has extended down and formed a subcutaneous mass. ("Virus Diseases" by Members of the Rockefeller Institute for Medical Research, Cornell University Press.)

then involve the principle of adaptation to a new host unaccustomed to the virus. One may also ponder upon the possibility of immunizing human beings against malignant neoplasms by treatment with tissue-juice from some human benign tumor bearing an as-yet-unknown relationship to cancer.

*Chicken Sarcoma.*—A neoplastic disease of chickens very much like human sarcoma (a kind of malignant growth) was described as early as 1911 by Rous.<sup>62</sup> It is known to be transmissible by filtrates of the sarcomatous tissues.

Although none of these infectious or transmissible animal tumors or "cancers" are known to have any relation to human disease, and although the human diseases are not known to be infectious,

the similarity is striking, and the analogy close. Yet the secret of human cancer still evades us.

**Virus Diseases of Plants.**—In spite of the fact that our knowledge of viruses began with the discovery of a plant virus in 1892, only since 1920 have virus diseases of plants been extensively



Fig. 367.—Symptoms on tomato fruits produced by infection with a plant virus. (Photo by McKay, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

studied. They are of enormous agricultural and economic importance.<sup>63, 64, 65</sup>

A great variety of agricultural and other plants is known to be affected by viruses. The diseases caused are commonly spoken of according to the type of symptoms or changes induced in the



plants. These changes may be classified under five main heads. (1) mottling and chlorosis; (2) distortion; (3) dwarfing and attenuation; (4) overgrowth; (5) necrosis.



A



B

Fig. 368.—A Symptoms of rugose mosaic. B Curly-top of sugar beet. (Photographs by McKay, Oreg. Agr. Exp. Sta., reprinted by permission from "Principles of Plant Pathology," by C. E. Owens, John Wiley & Sons, Inc., publishers.)

*Mottling* is due to disturbances in cell metabolism which result in differences in intensity of the green color of leaves, and often ex-

tends to fruits. This is characteristic of the mosaic diseases of various plants (tobacco, potato, tomato). The lighter areas are said to be chlorotic (Figs. 367 and 368, *A*). "Yellows" of peaches is a good example of chlorosis. Irregular coloration of flowers, often very beautiful in effect, and seen in tulips ("color break") especially is a symptom of virus disease.

*Distortion*, consisting of wrinkling, puckering, or cupping of the leaves, often accompanies virus disease. Distortion may also result in a rolling up or curling of the leaves. Mosaic diseases such



Fig. 369.—Tomato leaves showing extreme attenuation due to infection with a plant virus. (Photo by McKay from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

as rugose mosaic of potatoes, "curly-top" of sugar beets (Fig. 368, *B*) and cucumber mosaic show these symptoms very strikingly.

*Dwarfing or attenuation* is found in "witches broom" of potato and the mosaic of tomatoes (Fig. 369).

*Overgrowth*.—Tumor-like growths or "galls" are often caused by bacteria (see page 472), but also sometimes accompany infections by viruses. For example, tumor formation is seen in "winter blight" of potatoes and in the Fiji disease of sugar cane. In the latter, the galls occur in the stalks and leaves and consist of cells that are altered as to staining reactions.

*Necrosis*, or death of the parts affected, may occur in any of these diseases. Bacterial decay soon follows.

*Transmission of plant viruses* is accomplished much as is transmission of the bacterial diseases of plants. The viruses usually have to be introduced through the outer layers of cells, as by pricking or abrasions. Transmission by leaf-eating or leaf-piercing insects is therefore especially important in natural spread of these diseases.

The same methods of control apply to both types (virus and bacterial) of plant disease. Actual contact between diseased and healthy plants is to be avoided; biting insects are to be controlled; instruments and gloves are to be disinfected, and weeds are to be eradicated. Rotation of crops, use of clean or disinfected seed and avoidance of infected new stock or tubers are other means of control.

#### FILTERABLE FORMS OF BACTERIA

Some workers are of the opinion that minute forms (gonidia, etc.) develop during special reproductive processes of bacteria and that these are so tiny that, when released from the cell, they can pass through filters made of fine unglazed porcelain or infusorial earth (kieselguhr), or asbestos pads like the Gooch filters used in chemical laboratories. In this condition they are described as resembling, or actually belonging to, the group of *ultramicroscopic viruses*. Such forms are said by some to develop inside the bacterial cell wall by division of the cell contents without participation of the cell wall. It has been said, but never conclusively confirmed, that certain cultural conditions will induce the formation of virus-like forms by bacteria. The action of bacteriophage (see page 745) is also said to produce virus-like forms.

These possibilities cannot be denied but really convincing proof is nonexistent. On the other hand, a number of experimental studies make it appear highly improbable that bacteria are related to the viruses.<sup>66, 67</sup> Bacteria do not develop in virus diseases or in cultures of virus, as one would expect if the viruses were merely stages in the life cycle of bacteria. Many sources of error, which doubtless have misled some investigators, lie in the very complex problem of the physical properties of filters already referred to. It is evident that occasionally, even under the most carefully controlled conditions, when subjecting a culture of bacteria to the action of one of these filters, a bacterium may occasionally fail to be adsorbed and so appear in the filtrate and later multiply there, giving rise to the impression that some special type of filter-passing

granule was present in the original culture. The growth may equally well have resulted from the passage through the filter of an abnormally small cell, perhaps a young cell or dwarf variant but these can hardly be regarded as virus-like. It also may have resulted from accidental contamination of the filtrate or a minute defect in the filter, eventualities difficult to avoid.

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## CHAPTER 44

### BACTERIOPHAGE

ONE OF THE most interesting of the viruses is a parasite of bacteria. It was first described by Twort and later by d'Herelle and its action is often called the Twort-d'Herelle phenomenon. It is called *bacteriophage* (*bacterio* = bacterium; *phage* = eater).<sup>1</sup> Bacteriophage may be found in many situations in nature where bacteria are growing and is especially abundant in the intestine of man, animals and insects. In its resistance and several other properties bacteriophage resembles other plant viruses. Its action is quite different.

If a small amount of sewage, feces or ground-up flies or roaches be emulsified with broth and passed through a filter, the filtrate will often be found to contain this virus. It may be demonstrated by adding a drop or two of the filtrate to a very young, actively growing broth culture of appropriate bacteria. Dysentery or typhoid bacilli often serve.

Chicken feces or horse manure usually contains bacteriophage active against dysentery or typhoid bacilli. If, to a young, actively growing (early logarithmic phase) culture of these organisms, somewhat turbid to start with due to the presence of millions of bacilli, a few drops of bacteriologically sterile filtrate of the feces suspension are added, the culture will be found, after a few hours' incubation, to have become nearly or entirely clear and the live bacilli in it either reduced in numbers or entirely absent. If, now, this broth be filtered and a drop of the bacterium-free filtrate added to another young broth culture, this in turn will clear, and filtrates from it will induce the clearing phenomenon in other cultures. The lytic potency of the filtrates increases with each transfer, the bacteriophage multiplying enormously at each transfer and accomplishing the clearing much more rapidly and completely than at first.

If a loopful of the partly lysed (cleared) broth culture be smeared on an agar plate, any surviving bacteria that grow into colonies are often found to be distinct variants from the original, and it has been postulated that new species may originate in this way. The phenomenon is, indeed, very striking and offers interesting material for study. Smooth to rough variation is often induced in this way.

**Plaque Formation.**—Multiplication may also be made evident through an interesting phenomenon which may be produced by



smearing an agar plate with a drop of the young, active culture *before* putting in the bacteriophage, and then smearing another agar plate with the same culture a few minutes *after* the addition of the bacteriophage. The first plate will show profuse, normal growth, smooth and gray, unbroken except in the most thinly seeded areas where there are separate colonies. These will have smooth and regular edges. The second plate will show less growth and it will be dotted here and there with "pinholes" or places where no visible growth has occurred. These "pinholes" are called bacteriophage *plaques*. Each may be regarded as a "colony" consisting of billions of particles of bacteriophage, which has destroyed the bacteria around it. The number of plaques gives an idea of the number of bacteriophage-bearing particles in the original fluid culture (Fig. 370). Isolated bacterial colonies may show crescentic irregularities in their margins, as though pieces had been eaten out of the edges. They have been described as "moth-eaten" colonies.

The particulate nature of bacteriophage has long been under discussion. It has been possible to make electron photographs which show bodies believed to be bacteriophage (Fig. 371).

#### **Action of Bacteriophage.**—

Just how the bacteriophage acts upon the bacteria is not known. The dissolution of the bacteria seems to be entirely physical. At least, as shown by Ionesco-Mihaesti there is no hydrolysis of the cell protein as by an enzyme.<sup>2</sup> It is thought by some, following d'Herelle, that the bacteriophage particles penetrate into the bacterial cells and multiply there, causing the cell to burst and liberate increased numbers of bacteriophage bodies (Fig. 372). Others have amassed evidence to indicate that this is not the case. The exact mechanism of bacteriophage action is not settled. Electronographs seem to support d'Herelle's views.<sup>3, 4</sup> In any case the bacteria are actually destroyed in very short order, and the potency of the bacteriophage and the

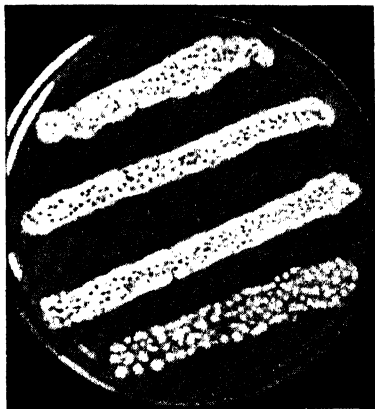


Fig. 370.—Bacteriophage action on the growth of a susceptible bacterium on the surface of an agar plate. The dark spots in the light bands are bacteriophage plaques. (After Caldwell, from Dible, "Recent Advances in Bacteriology.")

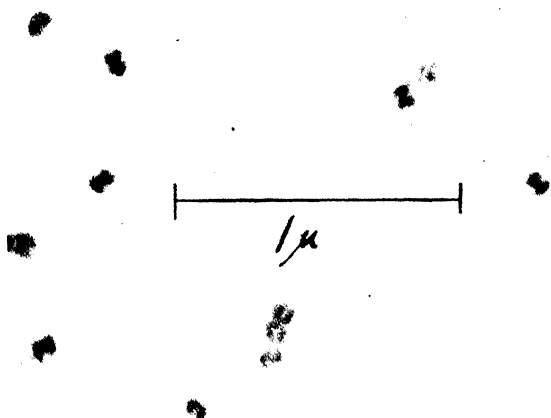


Fig. 371.—Electronograph of bacteriophage particles ( $\times 40,000$ ). Note the curious tail-like appendages and the apparently complex structure of the body (Luria, Delbrück and Anderson, Jour. of Bact., Vol. 46.)



Fig. 372.—Disintegration of a bacillus (*E. coli*) by the action of bacteriophage ( $\times 40,000$ ). (Luria, Delbrück and Anderson, Jour. of Bact., Vol. 46.)

number of plaques producible with a drop of filtrate may be greatly increased after lysis.

Like other viruses, bacteriophage will not multiply readily in any medium except living, bacterial cells. Dead cells, or dormant or old cells that are not *actively multiplying*, will not serve, although some experimenters seem to have obtained growth in dead cells or cell-free cultures under some conditions. Preferably young bacterial cultures are used. Bacteriophage will not *grow* in filtrates made from cultures which have been destroyed by the agent but will remain active in them for fairly long periods if refrigerated.

**Varieties of Bacteriophage.**—Bacteriophages active against various kinds of Eubacteriales have been found, but none has been found active against Thiobacteriales, Chlamydobacteriales, Spirochaetales or Myxobacteriales. There may be some that are active against certain of the Actinomycetales. However, the search has not been exhaustive. There is said, by some students of the subject, to be *only one bacteriophage* and that, by suitable adaptation, it can be made to attack various kinds of bacteria. Others explain this apparent adaptation as resulting from selective growth of one species of 'phage in a mixture, in contact with a pure culture of bacteria suitable to only one. This is a matter still in dispute.

**"Typing" of Species with Bacteriophage.**—The process of adaptation (or selective cultivation) of bacteriophage may be carried to a degree where a given strain of 'phage is selectively active, not merely on but a single species of bacterium but on only certain types or subdivisions of that single species. These subdivisions or types may be distinguishable solely by serological means, as in the case of *Shigella paradysenteriae* or, in the case of *E. typhosa*, by no other means except susceptibility to the highly specific 'phage. Certain kinds of hemolytic streptococci have also been extensively investigated in similar relation to bacteriophage susceptibility. Several details of these phenomena are of fundamental significance for bacteriology in general and may be discussed here.

**'Phage Types of *E. typhosa*.**—Craigie<sup>5</sup> and others observed that *E. typhosa* could be divided into two main subdivisions on the basis of 'phage susceptibility. The one, corresponding to rough variants, they designated as W. This variant is insusceptible. The other, corresponding to smooth, virulent variants, they designated as V. This is highly susceptible. Susceptibility of the V variants seems, further, to be closely related to content of Vi antigen. The smooth strains with less Vi antigen are less susceptible. Passage through several mouse infections rejuvenates both Vi antigen content and

'phage susceptibility. The reasons for the relationship between Vi antigen and specific 'phage susceptibility are obscure.

The relationship of 'phage susceptibility, Vi antigen, smoothness and virulence is undoubtedly of fundamental significance but requires further investigation. The general applicability of the principle to other species of bacteria of industrial, veterinary and botanical interest is obvious, and offers a good field for investigation.

In additional studies it was found that the V strains could be further subdivided into several distinct types on the basis of further adaptation of 'phage. By maintaining a given 'phage on a certain selected strain of V form of *E. typhosa*, the 'phage becomes so highly adapted or specific for that strain that it will not act (at least *not when diluted beyond a certain low concentration of the 'phage called the "critical dilution"*) on certain other V forms of the same species. In stronger dilutions the selective specificity is masked by an overwhelming action on all V forms. The first highly specific 'phage of this sort was designated as "'phage A" and the corresponding susceptible V form strain as "'phage type A of *E. typhosa*." By a like process several 'phage types of *E. typhosa* of the V form were discovered, and were designated by letters A, B, C, D, E, etc.

**Use of Bacteriophage Types.**—Having prepared several such highly selective and exclusive type 'phages and determined the *critical dilution* of each, studies were made by Craigie and others, and by Lazarus and others of the 'phage types of typhoid bacilli occurring in various patients and in epidemics.<sup>6, 7, 8</sup> The method consists in inoculating an agar plate in several areas with young culture of the epidemic typhoid organism to be tested, allowing a few hours' incubation, and then putting a loopful of the critical dilution of different types of 'phage in the center of each area and incubating until there is clear differentiation between the unaffected areas and the part lysed by the 'phage. The type 'phage producing a large, clear zone in the area of growth indicates the type of typhoid bacilli being dealt with (Fig. 373).

It was possible to show in some instances that the strains of *E. typhosa* in a group of patients were all of the same type and therefore probably came from the same source. It was possible to trace the source or sources and to discover carriers of the same type. In other epidemics, two types were found and traced to two different carriers.

**Bacteriophage Analysis of Genus Shigella.**—Related investigations were carried out by Thomen, of the Dominican Republic, a

highly skilled scientist.<sup>9</sup> This worker collected strains of important species of *Shigella*, including the serological variants of *S. paradyenteriae* (see section on *Shigella*, page 484), and also several species whose position in the genus is doubtful. By a long process of adaptive and selective cultivations of bacteriophage by methods analogous to those of Craigie and others, but differing in important technical details, he produced a series of 'phages of high differential

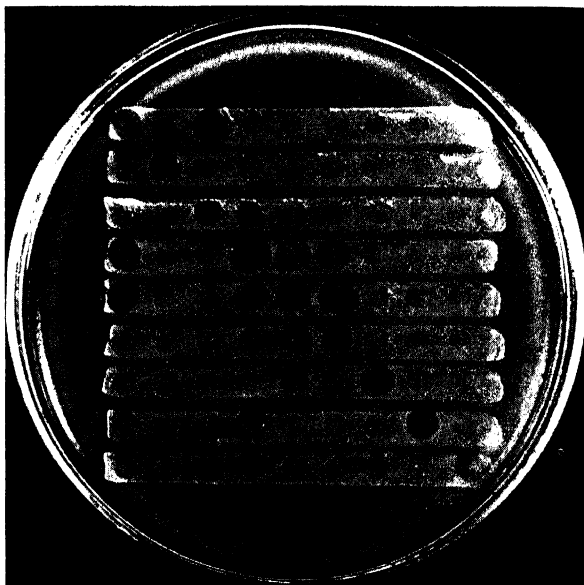


Fig. 373.—Large Petri plate containing agar with strips of growth of various species of *Shigella* (inoculated by means of small, sterile camel's hair brushes). Droplets of several different specific bacteriophages diluted to the critical point have been spaced along each strip. The completely cleared area produced by the homologous 'phage, and some partially cleared areas resulting from the action of related phages, are clearly seen. (Courtesy of Dr. L. F. Thomen, Secretary of State for Public Health, Republica Dominicana.)

potency with which an analysis of the relations, serological and cultural, between the various component species and varieties of the genus was made.

A number of important facts were brought to light. For example, it was shown that doubtful species (*S. gallinarum*, *S. psaffii*, *S. rettgeri* and others) were not readily lysed by any of the 'phages developed on true *Shigella*. Bacteriophage developed exclusively on

*S. ambigua* (a serologically and culturally distinct species) was highly specific and potent, while 'phage developed on any one of the serologically related races of *S. paradysenteriae* (which are differentiated poorly by cultural and serological tests) always cross-reacted to some extent with other races of the same species. A relationship between *S. sonnei* and *S. madampensis* and *S. ceylonensis* (culturally related species) was seen in a certain amount of cross reactions between these organisms and their respective bacteriophages.

The same procedure is applicable to other genera such as *Staphylococcus*<sup>10</sup> and possesses certain advantages over serological and cultural methods although, because of difficulty in developing absolute specificity, it may not replace them at present. Perhaps complete specificity may be later accomplished by more prolonged selective propagation. The method is rapid, no animals need be immunized, no confusing and expensive carbohydrate tests are needed, and the results seem to be related to serological and cultural properties at the same time.

*Bacteriophage in Relation to Streptococci.*—Evans,<sup>11</sup> at the National Institute of Health, by procedures similar to those described, developed bacteriophages highly specific for certain hemolytic streptococci following observations on this phenomenon by Lancefield.<sup>12</sup> Evans' studies emphasized the importance of what she termed "nascent 'phage." In the presence of the specific, sensitive culture on which any given 'phage was developed, the 'phage could attack many strains which were entirely resistant to the filtered 'phage *alone*. Thus, the range of activity of a given specific 'phage for other strains of bacteria may depend on whether the specific homologous culture is present or not.

Evans described four races of 'phage (A, B, C, and D) which differed in their activity (all tested in the nascent state) on different kinds of hemolytic streptococci and other organisms. For example, about 90 percent of beta hemolytic streptococci were sensitive to 'phage race A, but only about 10 percent to 'phage race D. These 'phages lysed all strains of pneumococci tested, but none of the strains of alpha-type streptococci examined. Thus a curious relationship between beta-type streptococci and pneumococci was revealed. The value and possibilities in such studies are made clearly evident.<sup>13, 14</sup>

**Therapeutic Use of Bacteriophage.**—When bacteriophage was first discovered, it was thought that disease could be cured with it. If, for example, persons with cholera could swallow cholera

“phage” or have it injected into them, it was thought that the phage would parasitize the cholera vibrios in the intestine and thus cure the disease. Similarly, it was thought that persons suffering from boils could have antistaphylococcus phage injected, or applied in compresses, with a resulting destruction of the staphylococci and cure of the boils. However, it was soon found that bacteriophage will not act in the presence of blood, pus, fecal or any colloidal material<sup>1, 15</sup> and hope of bacteriophage therapy has been largely abandoned in most quarters.

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## CHAPTER 45

### THE RICKETTSIAE

RICKETTS was first to call attention to a formerly unrecognized group of organisms which differ in many respects from any previously known. His earliest studies on the group were made in connection with Rocky Mountain spotted fever. In 1909 he first described the organisms and mode of transmission of that disease. A year later he discovered the organism of typhus fever while working in Mexico. During his studies of typhus\* in 1910 he contracted the disease and died. In 1916, da Rocha-Lima, a Brazilian scientist, made further observations on the organisms described by Ricketts and named them *Rickettsia* in honor of their discoverer. He also gave the name of *proWazekii* to the rickettsiae associated with typhus fever, in honor of another scientist, Prowazek, who had lost his life in the study of that disease.<sup>1</sup>

About fifty species of rickettsiae have since been described, a few in connection with human diseases. They occur principally as parasites in various insects but some are transmissible to human beings.

The rickettsiae constitute a little-understood group of organisms and there is difference of opinion as to whether they are bacteria, stages in the developmental cycle of viruses or neither of these but an entirely different class of living beings. Some even doubt their existence, believing them to represent cell debris. Most probably they are neither viruses nor bacteria, but an intermediate group. Morphologically they are intermediate between the large viruses, like the lymphogranuloma-psittacosis group, and the bacteria.

A great deal of experimental work has been done in connection with them and the diseases they cause. Assuming them to be living organisms, as the best evidence clearly indicates, we shall present here only their outstanding characteristics since a detailed account of them would necessitate an unduly prolonged discussion.<sup>2, 3, 3a, 4, 4a</sup>

**Characteristics of Rickettsiae.**—The organisms are very tiny, having diameters of about 0.3 micron and lengths seldom exceeding 2 microns and usually much less than this. They are variously shaped, like minute rods, diplococci and single cocci. Sometimes relatively long filaments are formed. No spores are produced.

\* Do not confuse typhus fever, a blood and tissue disease transmitted by lice and rat fleas, with typhoid fever, a predominantly intestinal infection transmitted by way of the mouth and due to *Eberthella typhosa*.



Unlike viruses, they are not filterable, and in this respect resemble bacteria. They are nonmotile and it is difficult to stain them with

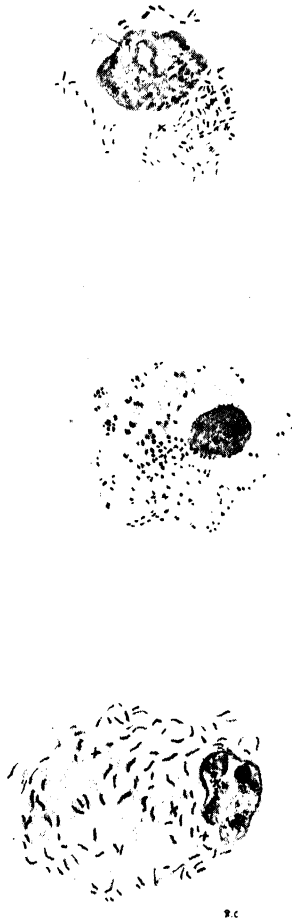


Fig. 374.—Rickettsiae in the cells of a guinea-pig. (Redrawn from Monteiro.)

ordinary dyes. They can, however, be colored with Giemsa's stain. They are gram-negative. The rod-forms tend to stain more intensely at the tips, somewhat like the pasteurellas (Fig. 374).

**Cultivation of Rickettsiae.**—A few are said to have been cultivated in special lifeless laboratory media but the majority do not multiply outside the living cells of animals or insects, being obligate intracellular parasites and, therefore, much resembling the ultra-microscopic viruses. Most workers would exclude artificially cultivated species from the group of true rickettsiae. Rickettsiae may be cultivated in live chicken embryos and in tissue cultures like those used to cultivate viruses (see section on methods of cultivation, page 161).<sup>5, 6, 7</sup>

**Habitat.**—The rickettsiae seem characteristically to inhabit the cells lining the intestines and other tissues of insects, both blood-sucking and non-blood-sucking. Human pathogenic species of rickettsiae inhabit insects which bite man or animals or both. Non-pathogenic rickettsiae have been found in ticks, fleas, lice, bedbugs, spiders and mosquitoes. Some varieties of the organisms are found in the lumen of the intestines of the insects but many are found only in the epithelial cells lining the intestine and in the salivary glands, whence they may be transmitted to man. Since they sometimes occur in the intestinal contents they may appear in the feces and transmission to animal hosts may be obtained by rubbing or scratching this material into the skin.

In the United States the most important diseases caused by rickettsiae are typhus fever and Rocky Mountain spotted fever. These two diseases illustrate some very interesting immunological relationships between themselves and between the rickettsiae and certain bacteria.

**Rickettsial Diseases.**—The human rickettsial diseases, except one known as Q fever, are clinically similar, characterized by fever, skin rashes or dark blotches due to lesions of the blood vessels, and involvements of the brain. However, there are also striking clinical differences between the various kinds of rickettsial diseases. They (and their respective causative rickettsiae) may be divided into three groups on the basis of clinical data and insect vectors. First, *R. orientalis* and related types of rickettsiae are the cause of a group of similar febrile diseases in Japan and adjacent lands called *tsutsugamushi* disease. These diseases are transmitted by the bites of larvae of mites occurring in rice fields and swampy areas. Second, Rocky Mountain spotted fever is typical of a second group of similar fevers. The organism involved in Rocky Mountain spotted fever (*R. rickettsii*) is transmitted by the bites of various ticks. Third, *R. prowazekii*, causing typhus fever, is transmitted to man by the bites and feces of body lice and feces of rat fleas. There are

several typhus-like diseases. The fourth disease (or group of diseases) is clinically distinct from typical cases of the other three. The conditions of this fourth group are included under the name Q fever and in many respects clinically resemble influenza or atypical pneumonia ("virus pneumonia"). Characteristically there is no rash and no Weil-Felix reaction (see page 756). Headache, night sweats, continued fever, and vague pains in the chest and back occur. The causative agent, *R. burneti* or *R. diaporica*, appears to differ from other rickettsiae in being filterable. Transmission may be by ticks or the infection may be air-borne. There is no fatality so far as is known.

Both man and certain animals are susceptible to diseases of all four groups, and various biting insect vectors, other than those specified, may transmit each, especially those of the typhus and Rocky Mountain spotted fever groups. Because of the existence of different vectors in different geographical areas, and because there are sometimes clinical differences and differences in geographical distribution, various names have been given to diseases of the three types in different parts of the world. For example the typho-exanthematico of Sao Paulo, Brazil, is probably the same disease as Rocky Mountain spotted fever of Montana. Tabardillo of Mexico is typhus, and mite fever of Sumatra is identical with, or closely related to, Japanese tsutsugamushi.

**Typhus Fever.**—This disease, due to *Rickettsia prowazekii*, has been known since very ancient times. The disease known today as *typhoid* fever was so named because of its resemblance to typhus fever, with which it was for a long time confused. Typhus is found in Central Europe and occurs also in South and Central America, Asia, Russia, Africa and in the United States. It has appeared in occupied countries of Europe since the present war began.<sup>8, 9</sup> It becomes epidemic whenever conditions are suitable for populations to become thoroughly infested with lice. Troops in trenches, seamen in old-time ships, and crowded, poverty-stricken peoples during war or famine are thus especially likely to be visited by epidemics of the disease. It may be comforting to know that lice also die of the infection. Both Napoleon and Hitler had cause to know "General Typhus" and "Corporal Louse" to their sorrow. Newly developed insecticides (DDT) are helping greatly in the prevention of lousiness.

Typhus fever is characterized by an onset of about two days during which there is nausea, headache, dizziness and high fever. There then appears a rash which may cover the whole body. It

lasts for a week or more and disappears slowly. The patient is lethargic and delirious (typhus is from a Greek word meaning stupor). The mortality is often high. The blood of patients is infectious but the organisms have not been seen in the blood.

*Murine (Rat) Typhus.*—The rickettsiae of typhus fever were found in rats (*murine* typhus) in the United States after investigations by Maxcy<sup>9a</sup> showed that louse transmission from man to man in this country was a negligible factor and that rats were undoubtedly a reservoir of the disease. It was shown that, analogous to plague, the disease was maintained among the rodents through the agency of rat fleas and rat lice. It is transmitted to man from rats by rat fleas (*Xenopsylla cheopis*) when, as in plague, rats and man live in close and continuous contact. Once established in man, the louse-to-man epidemic cycle is said to occur if conditions of lousiness are suitable. Mice and other rodents may become infected but it is not clear how important this is from the standpoint of human disease.

*Typhus in Europe.*—The military campaigns in Poland in 1939, Western Europe in 1940 and in the Balkans in 1941 probably resulted in little spread of typhus due to the brevity of the actions, warm weather, and relative low level of louse infestation and infection. In the winter campaigns of 1941–1942 and 1943 conditions changed and, with little bathing and little chance of removing, cleaning or changing clothing, due to continued fighting, winter cold, and movement of troops and prisoners across Europe in filthy conveyances, lousiness appeared and with it typhus, which spread widely in areas hitherto free from it—Germany, Occupied Europe, etc. The danger of pandemic typhus, with continued devastation of Europe, is not remote unless DDT is generously applied to kill lice.

**Weil-Felix Reaction.**—In attempts to determine the causative agent of typhus fever Wilson, in 1910, and Weil and Felix, in 1915, isolated various strains of *Proteus* bacilli from patients ill with the disease. It was found that the serum of typhus fever patients would agglutinate certain strains of the bacilli even when diluted 1 : 2000 or higher. One strain of *Proteus*, designated as “X19,” was especially easily agglutinable and was not readily agglutinated by serum from patients ill with other common diseases. The agglutination test with *Proteus* X19, therefore, became of great value in the diagnosis of typhus fever and is spoken of as the *Weil-Felix reaction*.<sup>10, 10a</sup>

It was later found that *Proteus* bacilli, including X19, produced

two variants: one, which spreads over agar media in a thin, gray, transparent veil-like growth, was called the *hauch* (German equivalent for *spreading*) or "H" form; the other, forming small, discrete, circular colonies much like other bacteria was designated the *ohne-hauch* (German equivalent for nonspreading) or "O" form. The O forms are without flagella, and contain only somatic antigens as opposed to the H forms which contain both somatic (O) and flagellar (H) antigens (see section on typhoid bacilli, page 483). The agglutination reaction with typhus fever serum was found to be active and specific only with the O form, so that strains of X19 used for diagnosis must be of the O variety (OX19).

*Nature of Weil-Felix Reaction.*—This curious relationship between *Proteus* bacilli and the rickettsial diseases has been the subject of a great deal of speculation and experimental research. Some have suggested that the *Proteus* bacilli are variant forms of rickettsiae, or vice versa; others, that the rickettsiae, *Proteus* and a filterable virus are stages in the life cycle of a single organism. The idea was held by some that *Proteus* bacilli became modified by contact with the rickettsiae so that they acquired new "receptors" for the rickettsia antibodies.

Actually, no final explanation is available. *Proteus* bacilli do not appear to have any etiological relation to the rickettsial diseases. They are only infrequently isolated from the patients; they do not produce the disease in guinea pigs, nor do they occur regularly in infected guinea pigs; *Proteus* strains do not immunize against rickettsiae, nor does recovery from rickettsial infection immunize against experimental *Proteus* infection.

The most likely explanation, offered by Zinsser and his associates, is that rickettsiae and certain *Proteus* strains contain some antigenic component in common, just as many animal tissues contain an antigen, the Forssman antigen, in common with sheep erythrocytes. The phenomenon of sharing of antigenic components is one frequently referred to in this book and is a common and important biological phenomenon. In support of this idea it may be noted that Castaneda was able to isolate "soluble specific substances" from both typhus rickettsiae and *Proteus* OX19 which were identical in their immunological reactions.<sup>11</sup>

**Rocky Mountain Spotted Fever.**—This disease, due to *Rickettsia rickettsii*, is similar to typhus fever and resembles it clinically in important respects (Fig. 375), but also differs, so that clinical distinction can be made in typical cases. There appear to be at least three forms of the disease, very closely related: the eastern form,

occurring in the Atlantic and other eastern states, less frequently fatal (25 percent), and transmitted chiefly by the dog tick *Derma-centor variabilis*; a more highly fatal (60–70 percent) western (Bitter Root Valley, Montana) form transmitted to man by the bite of both male and female sheep ticks (*Dermacentor andersoni*); and the Brazilian form (typho-exanthematico) transmitted by *Amblyomma cajennense*. The ticks occur infected in nature, probably maintaining the disease among dogs, rabbits and sheep by their bites, and, in the case of the western form at least, among themselves by transmission of the infectious agent from females to their progeny through the eggs and by sexual contact.<sup>11a</sup>

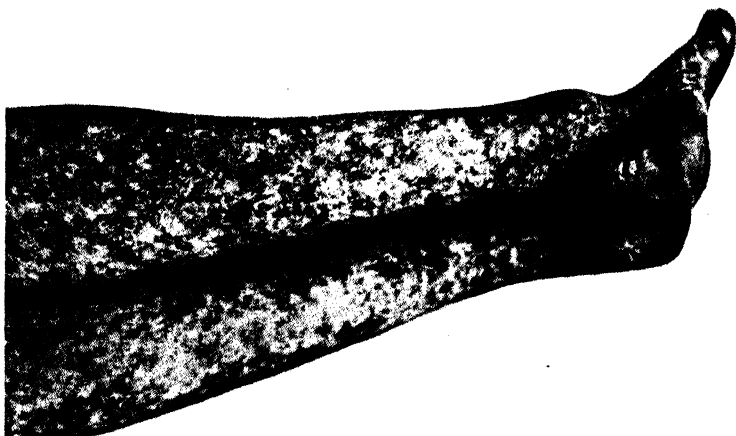


Fig. 375.—Fatal case of Rocky Mountain spotted fever. Photograph taken about the eighth day. Many patients in the Bitter Root Valley, Mont., die before the rash has developed to this stage. (Courtesy of Surg. L. D. Fricks.)

*Weil-Felix Reaction.*—In Rocky Mountain spotted fever, the Weil-Felix reaction is positive, but sometimes not so strongly as in typhus fever. Further, other strains of *Proteus* may be agglutinated as strongly as OX19, *e.g.*, OX2 and rarely a strain derived from X19 by variation and called the Kingsbury or OXK strain.

However, the three main groups of rickettsial diseases may be differentiated, roughly, according to whether they produce agglutinins *predominantly* for one or another of the three strains of proteus bacilli. These differences may be tabulated as follows:

TABLE XX

Variety of Proteus	Typhus Fever	Tsutsugamushi Group	Rocky Mountain and Related Fevers
OX19.....	+++	—	++
OX2.....	+	—	++
OXK.....	—	+++	—

The use of the Weil-Felix reaction *alone* to differentiate Rocky Mountain fever from typhus fever is not recommended. A much more reliable method is the complement-fixation test, using suspensions of the respective rickettsiae as antigens (see page 298).<sup>12, 13</sup>

*Avoidance of Spotted Fever.*—This is not difficult if one can follow four simple rules:

1. Stay out of tick-infested woods and fields, during May, June, July and August (North and Central states), especially where there is dense undergrowth overrun with wild mice, rabbits, etc.
2. If forced to travel in such areas wear high boots and white or light-colored clothes made of smooth, nonwoolly goods. Wear socks or boots outside of trouser legs because ticks tend to climb upward. They usually can be felt on the neck after a while.
3. Examine the clothing (inside and out) and body twice a day. If ticks are found, do not become unduly alarmed. It is unlikely that your tick is infectious; they do not bite immediately; even after biting they do not always infect immediately because the virus requires from two to eight hours' contact with your blood in the tick to become "activated" unless the tick has been warm for weeks or partly engorged on other animals, when it is already active. If the tick has taken hold, remove it and touch the area with iodine. Removal may be accomplished by gentle traction, unless the head is well embedded in the skin. Force may detach the head and leave it in the skin. With a fine forceps pinch up the epidermis, containing the tick's head, into a tiny fold and clip off just enough to remove the head. Disinfect the spot thoroughly. Do not crush or damage the tick or use bare hands.

4. Do not remove ticks from dogs or other animals or handle ticks from dogs or come into contact with their crushed bodies. Keep dogs outdoors in the tick season.

**Tsutsugamushi.**—This disease, caused by *R. orientalis*, is found principally in Japan, Malaya, and islands of the South Pacific.<sup>14</sup> It is primarily a disease of swamp and flood areas, but not all such areas are infected. The common transmitting agent (*Trombicula akamushi*) is much like the American "chigger." It is a mite of red-orange color, almost microscopic in size, which infests field mice and other animals in rural areas which are periodically flooded. The rodents serve as a reservoir for the disease. The larvae become infected from infected adults through the eggs and transmit the rickettsiae to man and animals by their bite. The adults do not bite mammals, but may live on plant juices. The disease is largely restricted ordinarily to low-class laborers and squatters who inhabit the areas described and is more common in males who labor in the fields.

The disease begins with a hard swelling or ulcer of the skin at the site of the bite. Then come fever, chills, a local ulceration, nausea, headache and the rash which, in one form or another, is found in most rickettsial diseases. The mortality may run as high as 60 percent, especially in older people. The serum of patients agglutinates only the OXK strain of *Proteus*. The hard swelling at the site of the bite is also found in *fièvre boutonneuse* and tick-typhus of South Africa, two diseases closely related to typhus fever, but tick-borne. The Weil-Felix reaction in these two diseases is like that in the Rocky Mountain fever group.

**Rickettsia Vaccines.**—One of the outstanding results of studies of Rocky Mountain spotted fever was the development of an effective and safe preventive vaccine by Spencer and Parker of the United States Public Health Service.<sup>15</sup> This is made by grinding up heavily infected ticks and treating extracts from them with 0.5 percent phenol to disinfect them and to inactivate the rickettsiae.

Another rickettsial vaccine (against typhus fever) has been prepared from formalinized rickettsiae obtained from tissue cultures of guinea pigs' testes.<sup>5</sup>

A method of cultivating Rocky Mountain spotted fever and typhus rickettsiae in embryo chicks entirely free from bacteria and insect material was developed by Bengtson and Dyer of the United States Public Health Service.<sup>7</sup> The method has been shown to be suitable for preparing rickettsial vaccines. A method of preparing typhus vaccine is based on this procedure, as described below.



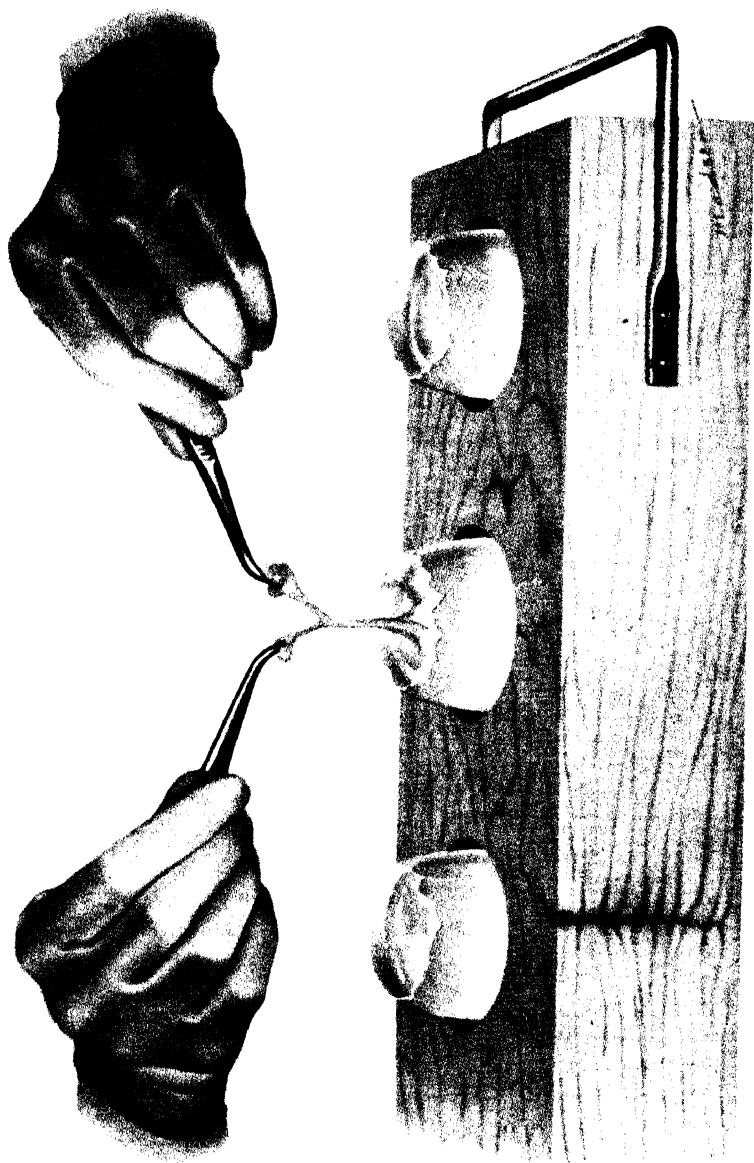


Fig. 376.—Harvesting yolk-sac membranes in the preparation of typhus vaccine. (Courtesy of E. R. Squibb & Sons.)

Because of the spread of typhus in Europe and Asia in 1942-44 extensive studies have been made of vaccination against the disease. Some of the vaccines tested have consisted of living rickettsiae of murine typhus, but these are falling into disuse because of the danger of spreading the disease and because the killed vaccines seem equally effective. Enthusiasm for typhus vaccines in general has not been great, because of the difficulty of demonstrating that they regularly offer protection, even though the vaccinated person may develop a positive Weil-Felix reaction. There are also considerable difficulties in the way of preparing the bulk of material necessary for extensive vaccination of whole populations, as in war-devastated countries.

One type of vaccine in favor in Europe is made from triturated stomachs of lice inoculated intrarectally with rickettsiae by means of a micropipette (Weigl's vaccine). This method is laborious, and is dangerous because of the possibility that infected lice may escape during the period when they are being incubated, fed, handled, etc. The rickettsiae multiply greatly in the lice. The gastro-intestinal tracts are removed, suspended in 1 percent phenol solution, triturated, and diluted to a final concentration of 0.5 percent phenol.

A typhus vaccine at present under trial in foreign field service under United States jurisdiction was originated by Cox, also of the United States Public Health Service.<sup>16</sup> The rickettsiae are injected into the yolk sac of developing chick embryos, where they multiply enormously (Fig. 376). The vaccine is prepared from either the whole embryology, or from the yolk sac alone, by grinding and adding 1.0 percent phenol and 0.5 percent formalin. Much of the solid tissue is removed by slow centrifugation of the ground-up embryo. The rickettsiae are then collected and washed by several high speed centrifugations.

These vaccines seem to give promising results,<sup>16a</sup> but certain, durable, and absolute protection of each individual must not be expected. Vaccines against other rickettsial diseases will doubtless be prepared on a large scale in the same way. Serum of immunized rabbits or goats may be useful for therapeutic purposes although extensive data are lacking as yet.<sup>17, 18</sup>

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## CHAPTER 46

### PLEUROPNEUMONIA AND RELATED ORGANISMS

DURING THE last decade there has been an increased interest in, and accumulation of knowledge of, a group of organisms definitely not protozoan or algal, yet difficult to classify as bacteria, viruses or rickettsiae, possessing some of the properties of all, yet not all the properties of any. The first member of the group to be discussed was demonstrated in 1898 by Nocard and Roux, who cultivated it on cell-free media from the serofibrinous exudate taken from the pleural cavity of a cow suffering from the disease now known as pleuropneumonia of cattle. Borrel afterward named the organism *Asterococcus mycoides*. In 1900, filterability of certain living pathogenic agents having recently been discovered (virus of tobacco mosaic—Ivanowsky, 1892) it was fashionable and “progressive” to test the filterability of all sorts of organisms and, when filtrates from pleuropneumonia cultures and tissues were tested, they were found to contain the organisms in viable form.

**Cultivation.**—Careful study of the causative agent of bovine pleuropneumonia and of several related types, since isolated, showed that growth will occur when pathological material is streaked on plates of suitable medium, even though no bodies resembling rickettsiae or ordinary bacteria are visible in Giemsa-stained smears of the inoculum. The most favorable medium was found to be heart-infusion peptone agar, pH about 7.7, containing about 25 percent serum. Horse, bovine, human or rabbit sera will serve but an organism once adapted to growth on the protein of one animal may sometimes be induced only with difficulty to utilize that of another animal. Media containing less than about 5 percent of serum will rarely support growth of pleuropneumonia organisms, evidence of a striking difference between these forms

and true bacteria. Plates or tubes of fluid medium may successfully be inoculated with minced infected tissue, exudate, swabbings, etc. Incubation is at 37° C. for from two to ten days or more and the atmosphere in the plates must be saturated with moisture. The organisms are facultative with regard to oxygen.

**Colonies.**—Minute colonies, so small as usually to be invisible to the naked eye and visible only with a hand lens or low power of the microscope, appear along the lines of inoculation on plates

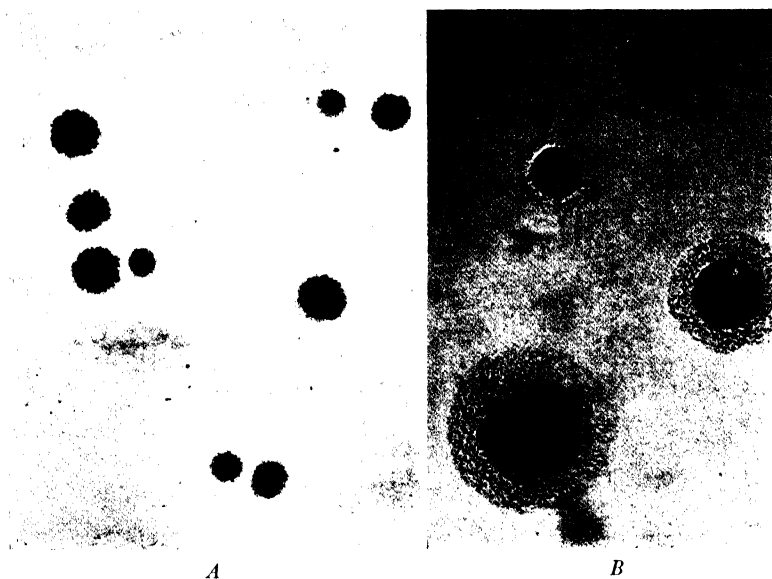


Fig. 377.—Colonies of pleuropneumonia-like organisms (L. bodies) on 20 percent horse-serum dextrose-starch agar. (Forty-eight hours at 37° C.) ( $\times 70$ .) Note the minute size and granular structure. *A* and *B* are from different cases of rat-bite fever. (Brown and Nunemaker, Bulletin of the Johns Hopkins Hosp. Vol. 70.)

(Fig. 377). These must not be confused with similar-looking structures ("pseudocolonies")<sup>1</sup> composed of sphero-crystals and other aggregations of material which sometimes appear on the surface even of sterile serum agar plates as a result of various physico-chemical factors acting locally, including scratching with a needle, and which have no relation to the inoculum except that scratches on the surface seem to facilitate their formation (Fig. 378). The genuine colonies are definite in outline and slightly raised and have a dark and vacuolated or granular central portion.

The agar containing such a colony may be cut out and used to inoculate fluid medium (25 percent serum-infusion-broth, pH 7.8 to 8.0) or other plates. Stained smears of such colonies do not contain recognizable bacteria.

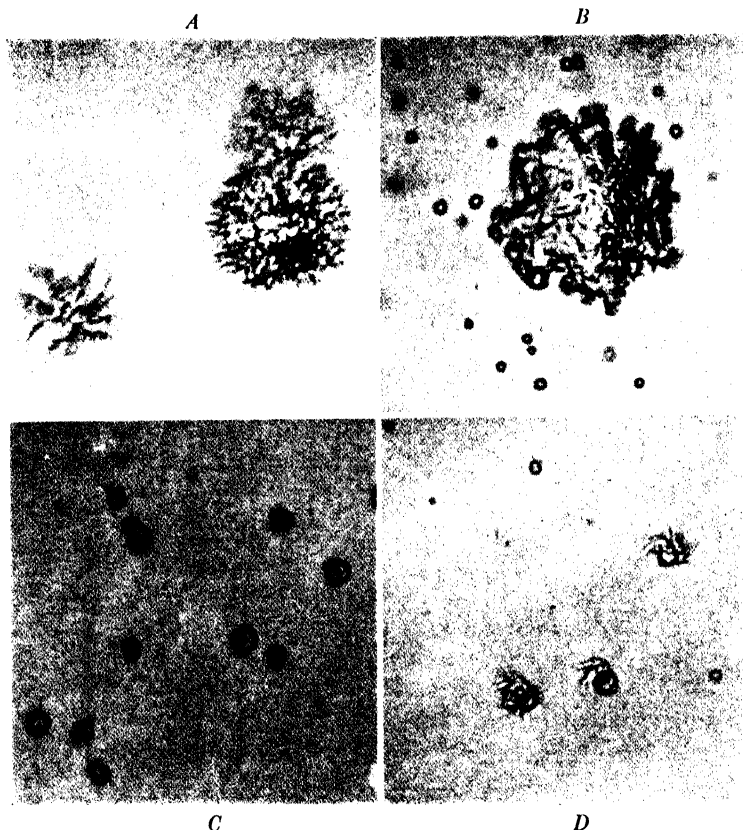


Fig. 378.—*A*, Pseudo-colonies in 30 percent rabbit serum agar, incubated two weeks.  $\times 115$ . *B*, Thirty percent rabbit serum agar, incubated six weeks. Pseudo-colonies have marked chiseled appearance.  $\times 115$ . *C*, Thirty percent ascitic fluid agar, incubated two weeks. Pseudo-colonies in form of small discs with central depression.  $\times 115$ . *D*, Thirty percent beef serum agar, incubated one week. Small central concave disc with curved peripheral areas forming cocks-comb pseudo-colonies.  $\times 115$ . (Brown, Swift and Watson, *Jour. of Bact.*, Vol. 40.)

**Filtration.**—In fluid medium fine turbidity usually appears after a few days although no ordinary bacteria are visible. It is with such material that filterability may be demonstrated, using collodion

membranes of suitable pore size or Berkefeld or other filters capable of withholding ordinary bacteria. Typical colonies grow on plates inoculated with the filtrate.

**Morphology.**—Studies of the morphology of pleuropneumonia-like organisms from various sources show that extreme pleomorphism is characteristic. Much depends on whether the material examined is from fluid or solid medium, its age, the conditions of incubation and various physical factors such as pH, surface tension, osmotic pressure, etc. Giemsa stain is preferable to others for examining the organisms. When Gram's stain is applied the result is poor, but the elements are gram-negative.

Apparently the cell protoplasm is not supported by very rigid cell walls and is easily distorted. In fluid medium one may see tiny coccoid or bacillary forms, curved or straight, chains of rings or combinations of any of these. Amorphous "blobs" and branching or filamentous bodies are also common in some forms. Material from solid medium is often so distorted and confused as to yield little information of value with respect to detailed morphological studies.

The exact mechanism by which the various forms described are produced is not entirely clear, but apparently some sort of fragmentation or fission occurs, perhaps in cysts, during which particles are formed so minute, or so different, from the ordinary bacteria as to be filterable.<sup>2, 3</sup>

**Antigenic Relations.**—Pleuropneumonia-like organisms from different sources are antigenically active and their immunological relationships have been extensively studied. Injection of cultures into rabbits induces the formation of agglutinins and other antibodies by means of which serological groupings, etc., have been investigated. The strains from bovine pleuropneumonia are serologically uniform and unrelated antigenically to any other group of similar organisms such as those from agalactia (cessation of lactation) of sheep, or from dogs affected by distemper, or from mice, rats, sewage and many other sources. The several groups of organisms are serologically separable and are also subdivided into subgroups.<sup>4</sup>

**Fermentations.**—In addition to serological group differentiations certain divisions are possible on the basis of fermentative properties. Thus, the organism of pleuropneumonia bovis is said to differ from that of agalactia in that the former fails to ferment sucrose while the latter causes fermentation. Both ferment maltose, differing from all other strains in this respect, while all known

strains ferment dextrose but fail to ferment sucrose except as noted.<sup>5</sup> (See Table XXI.)

TABLE XXI

## FERMENTATIVE PROPERTIES OF SOME PLEUROPNEUMONIA-LIKE ORGANISMS

Source	Lactose	Dextrose	Sucrose	Maltose
<i>Pl. bovis</i> .....	+	+	—	+
<i>Agalactia</i> .....	±	+	+	+
Others (rats, mice, etc.)	Variable	+	—	—

The fermentative studies are attended by numerous technical difficulties.

**Resistance.**—In respect to resistance to various unfavorable agents, this group of organisms resembles the nonspore-forming bacteria in many respects, and true viruses in others. Some are very sensitive to heat, 45° C. for fifteen minutes proving lethal to some strains isolated from mice, while other varieties, as that from *agalactia*, may survive seven but not ten minutes at 53° C. One strain, isolated from rats, may resist 56° C. for nearly an hour. Resistance of most forms to phenol, hydrogen peroxide and ultra-violet light is of a degree comparable with that of ordinary bacteria. They may be preserved for weeks or months by freezing and drying and will remain alive for a month or more on solid media sealed to prevent drying.

**Varieties of Pleuropneumonia-like Organisms.**—Following its discovery by Nocard and Roux in 1898 relatively little attention was given to this group of organisms. The cause of bovine pleuropneumonia was generally classed with the then mysterious and little known “filterable viruses,” the latter term at that time and even now, a sort of bacteriological wastebasket containing slightly studied agents of disease, some of them not viruses at all.

*Agalactia* is a disease principally of sheep and goats and causes an inflammatory disease of the mammary glands of lactating females which stop secreting milk—hence the name of the disease. The lesions of *agalactia* are not confined to the mammary glands however, the infectious agent attacking especially the joints and occurring in secretions of the eyes and in the blood. In 1923 Bridre and Donatien cultivated from the joint fluid of an infected sheep an organism very closely resembling that discovered by Nocard and Roux. It differs from the latter only in its pathogenic and serological properties.



*Asterococcus canis*.—A similar infectious agent was isolated by Shoetensack in 1934 from nasal discharges, lung exudate, etc., of dogs suffering from distemper. The relationship of the agent to distemper is obscure, as the disease is caused by a now well-known filterable (noncultivable) virus. Possibly there is a symbiotic activity. Shoetensack called his organism *Asterococcus canis* because of its apparent similarity to *Asterococcus mycoides*, the organism of bovine pleuropneumonia.

*Streptobacillus moniliformis*.—One of the most interesting members of this curious group of organisms is generally referred to as "L<sub>1</sub>" following the nomenclature of Klieneberger<sup>6</sup> who first described it in 1935. It is present in all cultures of the gram-negative, very pleomorphic, somewhat actinomyces-like bacteria usually called *Streptobacillus moniliformis* which occurs in the mouth and nares of wild rats. The streptobacillus is believed by some to be merely a variant form of the L<sub>1</sub> organism with which it is associated.<sup>7</sup> The name *Asterococcus muris* has been proposed for L<sub>1</sub>.

*S. moniliformis* and the L<sub>1</sub> organism grow well on 20 percent horse-serum infusion agar under the same conditions as other *Asterococcus* organisms. *S. moniliformis* may be obtained from pus of the ears of rats where it frequently causes otitis media, and from the blood of human beings suffering from rat-bite fever, one type of which it also causes.<sup>8-10</sup> The colonies, superficially examined, are much like those of ordinary bacteria. Smears show gram-negative rods and filaments in which branching is common. Swellings, globules and granulations of the cells are a prominent feature as the colonies age. The globules contain smaller elements which may be liberated by crushing under a coverslip (Fig. 379).

"L<sub>1</sub> Bodies."—After several days of incubation of the colonies, dense areas appear under them and, by washing away the earlier growth of *S. moniliformis* with broth, the denser portions are found to remain adherent to the agar. These are the growths of the L<sub>1</sub> elements. The agar containing these elements may be cut out, macerated with broth and used to inoculate new agar or broth. On agar they tend to retain their L<sub>1</sub> form; in broth the filamentous growth of *S. moniliformis* often reappears but not always. A great question remains unsettled as to whether the two forms are variants of each other, or whether they are distinct species. One view is that L<sub>1</sub> organisms are analogous to the pleuropneumonia organisms, but that in the latter the ability to form the filamentous growth like *S. moniliformis* is permanently lost (or was never developed). In the L<sub>1</sub> forms, according to this view, it is still present or represents

a slightly more advanced evolutionary phase which is more marked and persistent than in others. Any student of the group will find here a fascinating problem, challenging the objectivity of his judgment, his technical skill and his experimental ingenuity.



Fig. 379.—Various forms of *Streptobacillus moniliformis*. (Brown and Nunemaker, Bulletin of Johns Hopkins Hosp., Vol. 70.)

The  $L_1$  organisms or elements, as we have seen, grow more slowly than the *S. moniliformis* colonies. The colonies are very tiny and contain spherical bodies filled with granules which may be liberated

by crushing. They, as well as the globules in *S. moniliformis* filaments, are easily distorted and destroyed by changes in osmotic pressure, and lowered surface tension. The granules appear to be reproductive bodies, forming new, granule-filled, spherical bodies which burst and liberate their contents in turn. Similar phenomena have been described in *Asterococcus mycoides*.

The elementary granules, or some other reproductive form, will pass Berkefeld V filters, and *S. moniliformis* colonies will sometimes develop from such filtrates. However, before one could be certain that *S. moniliformis* developed from the filter-passing  $L_1$  reproductive units, one would have to be very sure of the integrity and fineness of his filters. The  $L_1$  bodies pass through fine collodion membranes. True bacteria do not pass such filters.

**Pathogenic Properties.**—Among many other interesting general details, which cannot be completely dealt with here, are the pathogenic properties of the pleuropneumonia group. There is, for example, good evidence that *Asterococcus mycoides* and a similar organism obtained from mice, produce an exotoxin somewhat like diphtheria toxin (compare with lymphogranuloma organisms).

A curious feature of many of the pathogenic forms is their affinity for joints and eyes. Some have repeatedly been isolated from animals suffering from arthritis.<sup>11, 12</sup> No relation between human arthritis or rheumatism and this group of organisms has been satisfactorily demonstrated. They may also infect numerous unrelated tissues. Each species seems to be definitely restricted to one animal species although the restriction is not absolute. Organisms of the group appear to be fairly widely distributed.

**Other Varieties.**—Certain lung diseases, pyogenic conditions and arthritis of rats appear to be due to members of the group, some of which have been called " $L_3$ ," " $L_4$ ," " $L_5$ ," " $L_6$ ," etc. In addition, an " $L_2$ " strain was described in guinea pigs. Forms indistinguishable from the pleuropneumonia group have been obtained from cultures of gonococci and from patients with gonorrhea.<sup>13</sup> They are said also to have been found in cultures of *H. influenzae*<sup>14</sup> and *E. coli*. In Europe nonpathogenic, apparently saprophytic, forms have been isolated from London sewage, by Laidlaw and Elford, and from soil, leaf mold and dung.<sup>15</sup>

**Classification.**—A survey of the properties of the filterable organisms of the pleuropneumonia group shows that they constitute a race apart. Obviously they are not bacteria in the sense of our definition of the Schizomycetes. They multiply by methods other than binary fission or budding and cannot be fitted morpho-

ogically into any of the present orders of bacteria. They and their colonies are exceedingly minute. They stain best by Giemsa's method. They are filterable. However, they resemble bacteria in being visible and cultivable on lifeless media, and in resistance to certain destructive agents. Some of them seem to produce toxins like bacteria.

They resemble viruses in size and filterability, and to some extent in pathogenicity but differ in ability to grow on lifeless media. The existence of saprophytic forms definitely excludes them from classification with true viruses. A few appear to grow intracellularly but so do some bacteria.

Certain phases in the development of pleuropneumonia organisms at times bear some morphological resemblance to some forms of rickettsiae, but there the resemblance between the two ends. The rickettsiae are neither highly pleomorphic like the pleuropneumonia organisms, nor are they readily filterable or cultivable on lifeless media or in the absence of living cells.

Pleuropneumonia organisms are not related to the filterable forms of ordinary bacteria (Eubacteriales) which have previously been discussed. The very existence of the filterable bacteria is in considerable doubt. Even if they do exist they are not visible and are not cultivable in the way the pleuropneumonia organisms are. The latter do not revert to ordinary bacillary forms unless *Streptobacillus moniliformis* be regarded as an exception.

There is, in view of the discovery of pleuropneumonia-like organisms in pure cultures of gonococci, and in several other bacteria, the possibility that they may exist as a variant form of all bacteria, in which case a complete revision of our notions of the life cycles, mode of multiplication, metabolism and many other factors concerning the Schizomycetes would have to be made. There is obviously a wide field for the student of general bacteriology, but his technic must be impeccable.

It seems proper, for the time being, to regard the group as another class of organisms, intermediate between the others. Sabin<sup>2</sup> has proposed the name Paramycetes for the group. We may console ourselves with the thought that the system of animate nature is in some ways analogous to the system of vibratory impulses or wave-motions. By special means, natural and artificial, we mark upon the spectrums of vibration frequencies or wavelengths certain divisions which conveniently demark pitches of audible sound, colors of visible light, bactericidal waves, high and low frequency radio waves, and so on, but each is merely a de-

tectable segment of a continuous whole. The intermediate gradations, though present, are not all perceptible to our senses or present instruments. So with living things, we classify the known forms into convenient groupings, but suspect that there are, or have been, intermediate forms, not readily assignable to any of the present artificial divisions. Occasionally one of these "connecting links" is actually demonstrated and a new grouping becomes necessary.

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## THE PATHOGENIC PROTOZOA

PROTOZOA are unicellular animals. They hold a position in the animal kingdom analogous to that held in the vegetable kingdom by the bacteria. Although microscopic in size, they are, in general, much larger than the bacteria and their structure and physiological activities are much more complicated than are those of the single-celled plants. Hundreds of kinds of free-living protozoa are to be found in much the same situations in the outside world as the numerous kinds of harmless bacteria. They are common in stagnant pools of fresh water, soil, feces, etc. Infusions of hay incubated a few days usually teem not only with bacteria but with protozoa. Leeuwenhoek was first to observe many of these. Only a few (possibly a score) kinds of protozoa are harmful to man. We shall describe the more important of these very briefly in this chapter. Students interested in learning more about protozoa should consult a book on protozoology.<sup>1, 2</sup> Many are of great importance in water and sewage purification, soil fertility and plant and animal disease.

**Structure and Biology of Protozoa.**—In protozoa there is always a well-defined nucleus which can be clearly seen with the microscope. There is also a definite cytoplasm and cell wall, and there are usually many small granules, and vacuoles filled with clear fluid. These have various functions connected with motion, digestion and reproduction. The specialized portions of the cell are called “cell organs” or organelles.

**Classification of Protozoa.**—All of the protozoan parasites which we shall discuss are motile during at least one stage of their existence, the means of motility being different in each group. These differences are used as a basis of classification. There are four great groups of protozoa and one or more species in each group is pathogenic. The groups may be listed, with one or two common examples, as follows:

CLASS I. *Sarcodina* (move with pseudopods).

A. *Endamoeba histolytica*—causes amebiasis.

B. *Endamoeba coli*—not pathogenic (?)

CLASS II. *Mastigophora* (move with long, whiplike lashes called flagella).

A. Intestinal tract:

(a) *Giardia lamblia*—causes enteritis (?)

(b) *Trichomonas hominis*—doubtful pathogenicity.

## B. Genital tract:

- (a) *Trichomonas foetus*—may cause disease in cattle.
- (b) *Trichomonas vaginalis*—may cause vaginitis.

## C. Blood parasites:

- (a) *Trypanosoma rhodesiense*—causes African sleeping sickness.
- (b) *Trypanosoma gambiense*—causes African sleeping sickness.
- (c) *Trypanosoma equiperdum*—causes dourine ("equine syphilis") of horses.
- (d) *Trypanosoma cruzi*—causes Chagas' disease in Brazil.

## D. Blood and Tissues:

- (a) *Leishmania donovani*—causes kala-azar.
- (b) *Leishmania tropica*—causes oriental sore.
- (c) *Leishmania braziliensis*—causes espundia or South American leishmaniasis.

CLASS III. *Sporozoa* (move with pseudopods only in immature stages; male gamete is flagellate).

- A. *Plasmodium vivax* (also *P. malariae*, *P. falciparum* and *P. ovale*)—cause human malaria.

CLASS IV. *Ciliata* (move with cilia).

- A. *Balantidium coli*—causes enteritis and ulcerations.

Some authors include a fifth class, Suctoria, in which young stages are ciliated while adult stages are provided with tentacles. None of these is known to be pathogenic for human beings.

**Reproduction and Life Cycles of Protozoa.**—Some protozoa multiply both sexually and asexually, others only by binary fission. Unlike the bacteria, the flagellates divide lengthwise (longitudinal fission) rather than crosswise. Ciliates divide transversely. The nucleus undergoes changes very much like mitosis. Some species exhibit a simplified or primitive form of this mitotic phenomenon. It is spoken of as promitosis.

Sexual multiplication by protozoa often involves rather complicated procedures. Many protozoa, during their development, pass through a definite and readily demonstrable series of stages and are thus quite different from the bacteria. These series of developmental stages are called *life cycles* and often are exceedingly complicated. In the case of the malarial parasite and some other protozoa, especially those which live in the blood, it is necessary for the parasite in its various stages to live in one or more insect hosts before it is mature and ready to infect man or animal again. The cycle involving sexual reproduction usually takes place in one host (the definitive host) while the asexual development occurs in another (intermediate host). This phenomenon of the developmental cycles is spoken of as *alternation of generations*. In the case of the malarial parasite the definitive host is a certain kind of

mosquito, while the intermediate host is man. In the trypanosomes the invertebrate hosts may be tsetse flies and other insects, the host depending on the species of parasite.

### CLASS SPOROZOA; THE PLASMODIA

**Malaria.**—Malaria is found in all parts of the world having tropical or temperate climates and in some places it has made human habitation impossible, as in Panama a century ago and in the Pontine marshes in Italy for many centuries. Recent campaigns against malaria, however, have redeemed many such localities. Nevertheless it still remains one of the most widespread of the communicable diseases.

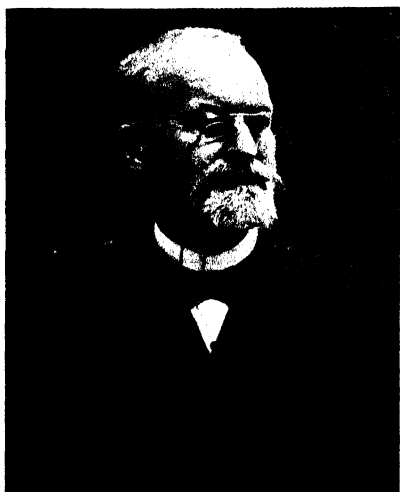


Fig. 380.—Alphonse Laveran.

The malaria parasite is an extremely interesting one and has been the subject of a great deal of study. It was discovered by Laveran, a French military surgeon in Algiers, in 1881 and his discovery has affected the lives of many millions of people and the destiny of nations (Fig. 380).

Ronald Ross, in 1898, during investigations of bird malaria, which is common, discovered mosquito transmission (by a *Culex* mosquito), while Grassi and Bignami dis-

covered, in the same year, that human malaria is transmitted by *Anopheles* mosquitoes.

The human parasites belong to the class Sporozoa, genus *Plasmodium*. There are four species: *Plasmodium vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. Parasites are also found causing malaria of birds and of monkeys. These are not known to infect human beings. A common bird parasite is *Plasmodium cathe-merium*; *P. knowlesi* is found in monkeys. These are of great importance in experimental studies.<sup>3, 4</sup> All have similar life cycles.

**Human Cycle (Schizogony or Asexual Generation).**—The infected malaria mosquito, during its bite, conveys the parasites into the blood of its victim with its saliva. The parasite in this stage is



called a *sporozoite*. The sporozoites soon attack red blood corpuscles and begin to grow within them. The parasite, inside of the corpuscle, moves in an ameboid manner which, in *P. vivax*, is especially lively, hence the species name. Malarial pigment is formed within the parasite. The parasite is now called a *trophozoite*, at first assuming, roughly, the shape of a signet ring ("ring-stage") with the nucleus as the seal (Fig. 381). This develops into the "ameboid stage." Each trophozoite multiplies by division,

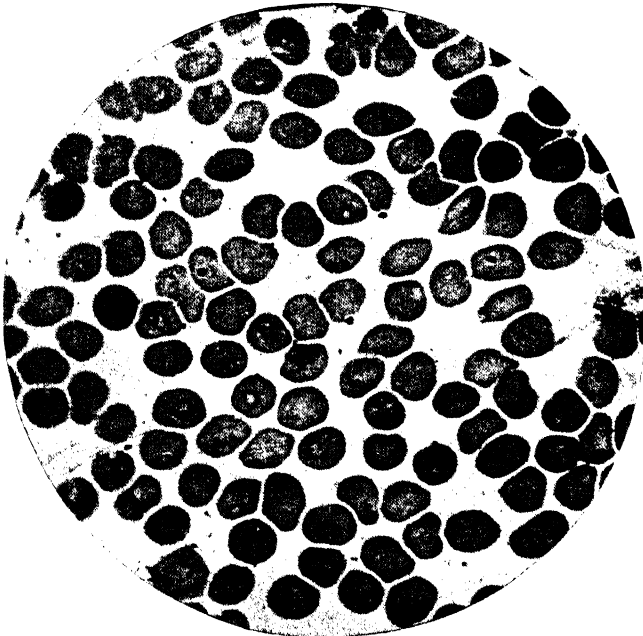


Fig. 381.—Smear of blood from malaria patient showing numerous ringlike trophozoites in the red blood cells.

forming a number of small bodies or segments which become arranged in a rosette. It is now known as a *schizont*. Eventually the affected corpuscle ruptures and the segments, along with pigments or metabolic products of the parasites, are liberated into the circulating blood. The patient at this time has a febrile attack often accompanied by a chill. The pigments are deposited in various tissues which eventually become deeply pigmented. Each segment is a new, active parasite and is now called a *merozoite*. As soon as it gets out into the blood, it attacks a new red corpuscle and in turn

multiplies and so the asexual cycle, or schizogony, is renewed. In this way the blood is soon filled with the organisms. The patient becomes anemic and weakened due to the destruction of the red corpuscles and possibly by poisonous metabolic products of the parasites.

The successive crops of parasites mature in the blood in great groups, all the individuals of which burst out of the corpuscles at about the same time. These crops mature at different rates, two, three or four days, depending on the parasite involved. This causes the successive "chills and fever." A febrile attack in a malaria patient means that a fresh crop of parasites has entered the circulation.

After a few cycles of this sort (five to ten days), while most of the merozoites continue to seek new corpuscles, certain others become differentiated from the majority of merozoites. They grow into mature, adult, sexual forms or gametes, which are large and almost entirely fill the erythrocytes in which they develop. Malariologists can distinguish the male and female gametes by their staining properties (Wright's or Giemsa's stain) and morphology. They float about in the blood stream and eventually perish unless taken up by an *Anopheles* mosquito. Their true nature then becomes apparent.

**Development in the Mosquito (Sporogony or Sexual Generation).**—MacCallum, the first to realize the true nature of the mature forms of similar parasites (*Halteridium*) in bird malaria, states, concerning these gametocytes: "Some remain quiescent; others, after violent convulsions of the protoplasm, throw out long, active flagella which beat about and soon become separate, free-swimming threads, like spermatozoa. These make their way to the quiescent forms, and of the little swarm which hovers about each of these female (quiescent) forms, or macrogametes, it is seen that one and only one buries itself in the protoplasm, while the rest perish. The flagella, or microgametes, are really analogous to spermatozoa." The fertilized form of the malarial parasite is called an *oökinete*. "Sir Ronald Ross, in India, discovered that there appeared pigmented cysts in the walls of those mosquitoes which have bitten persons ill with malaria, and formed the idea that the process of fertilization and formation of a motile zygote (*oökinete*) described above must occur in the mosquito's stomach, and that the development of cysts in the walls of that organ must be due to the fact that this new, active zygote (*oökinete*) could push its way into that situation and there become encapsulated."<sup>6</sup> Within these

cysts or capsules (called *oöcysts*) further divisions of the fertilized parasitic cells take place and the cysts burst, liberating enormous numbers of sporozoites, some of which migrate to the salivary glands of the insects and are ready to infect man and begin the

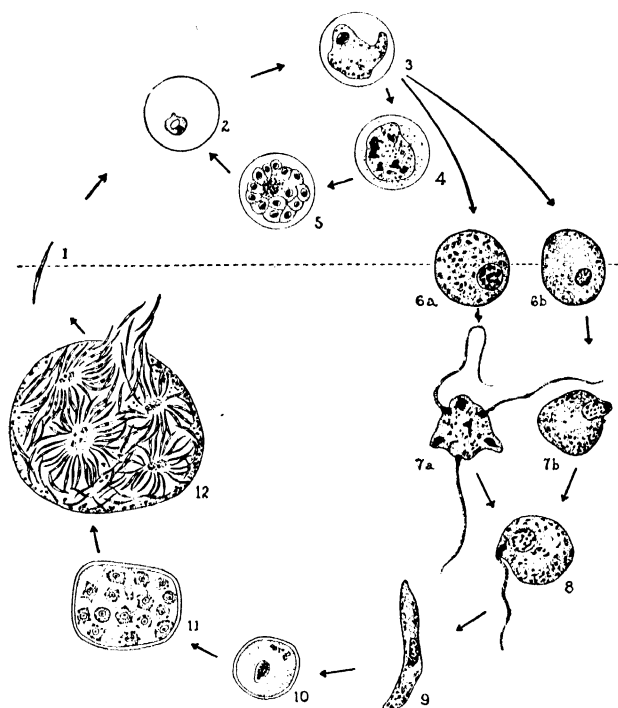


Fig. 382.—Life cycle of the tertian malarial parasite, *Plasmodium vivax*. The stages above the dotted line occur in the peripheral blood of man, whereas those below are found only in the mosquito. 1, Sporozoite; 2, trophozoite, in red cell; 3, full-grown schizont; 4, schizont with chromatin in several masses; 5, segmentation stage; 6a, male gametocyte; 6b, female gametocyte; 7a, exflagellation of male gametocyte—formation of microgametes; 7b, female gametocyte extruding chromatin from nucleus; 8, fertilization of macrogamete by microgamete; 9, ookinete; 10, young oöcyst; 11, oöcyst with many nuclei; 12, ripe oöcyst discharging sporozoites. (After Hegner and Cort from Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

asexual cycle or schizogony again (Fig. 382). The period between the ingestion of blood by a mosquito and the time when the bite becomes infective is usually around twelve days at temperatures of 25° to 30° C.

**Four Species of Malarial Parasite.**—One of the commonest species of the malarial parasite is *Plasmodium vivax*. Schizogony, in this species, requires forty-eight hours for completion, so that febrile attacks occur at intervals of forty-eight hours or every third day. This type of malaria is called *tertian fever*.

*Plasmodium ovale* causes a disease very much like malaria due to *P. vivax*, but milder. The two species are much alike.

Another species of the parasite, *P. malariae*, requires seventy-two hours for completion of the asexual generation and groups of parasites mature every fourth day. This form of malaria is called *quartan fever*. The ameboid stage is often represented by a "band form," which extends across the erythrocyte centrally and is characteristic of this type of infection.

The fourth species of parasite is called *P. falciparum*, due to its sickle-shaped gametocytes, and matures at irregular intervals between twenty-four and forty-eight hours. Temperature and chills appear, therefore, at irregular intervals. Only the ring stages and gametes are seen in the blood, other stages occurring in the capillaries. Often several rings are seen in the same red cell. This type of malaria is called malignant subtertian or *estivo-autumnal fever* because it commonly occurs in the late summer and fall. It is apt to be more severe than the other forms of malaria and appears to be more refractory to quinine treatment. A large number of the fatal cases of malaria are caused by *P. falciparum*. Relapses after apparent cure, especially in relation to changes of climate or season, are characteristic of malaria of all types.

**The Malaria-transmitting Mosquito.**—The name of the genus of mosquitoes which transmits malaria is *Anopheles*. A number of species of this genus are capable of transmitting the disease, some being more efficient in this respect than others. Common species are *A. quadrimaculatus* (especially in the southern United States), *A. maculipennis* (on the Pacific coast) and *A. gambiae* (the most dangerous vector in tropical Africa and, until recently, in Brazil).<sup>6, 7</sup> All have certain outstanding characteristics in common.

Only the female of any type of mosquito is equipped with biting organs. The female *Anopheles* may be differentiated from other kinds of mosquito by her position during a bite. This is indicated in Figure 383. The common *Culex* mosquito stands as shown in Figure 383. *Anopheles* mosquitoes can usually also be recognized by the presence of silvery or gray spots on the wings and often gray bands on the legs.

All mosquitoes pass through four stages of development. These are: the egg; the larva or "wiggler"; the pupa or "chrysalis" and

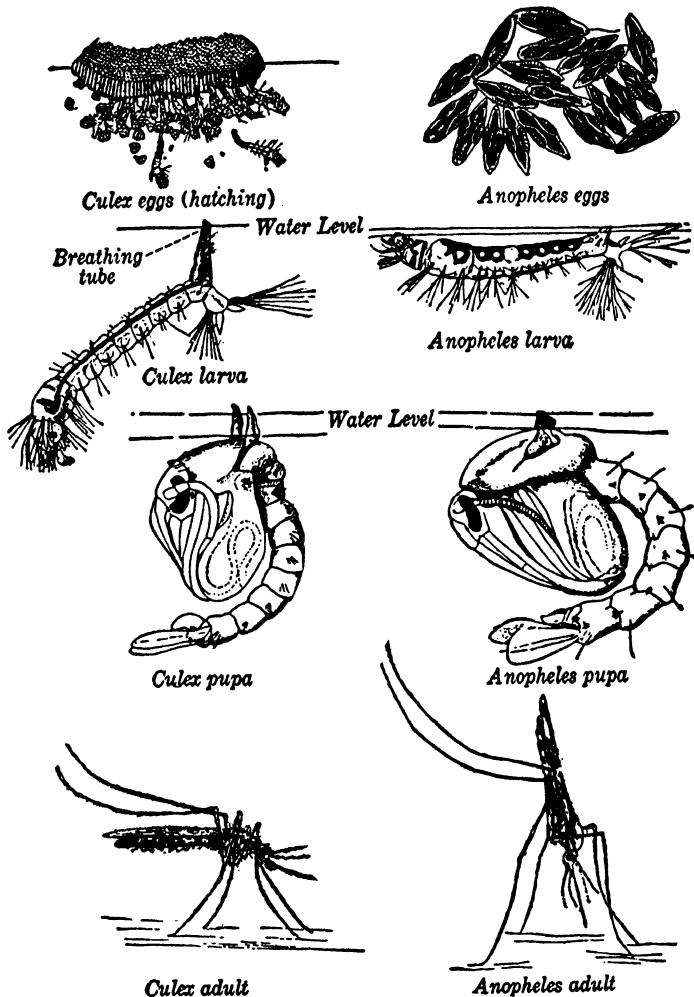


Fig. 383.—Mosquito. Stages in the life cycle of a common house mosquito of the genus *Culex* at the left and of a malaria mosquito, *Anopheles*, at the right. (Courtesy of U. S. Bureau of Entomology.)

finally the fully developed insect or imago. The first three stages are aquatic. *Anopheles* mosquitoes may be identified by certain

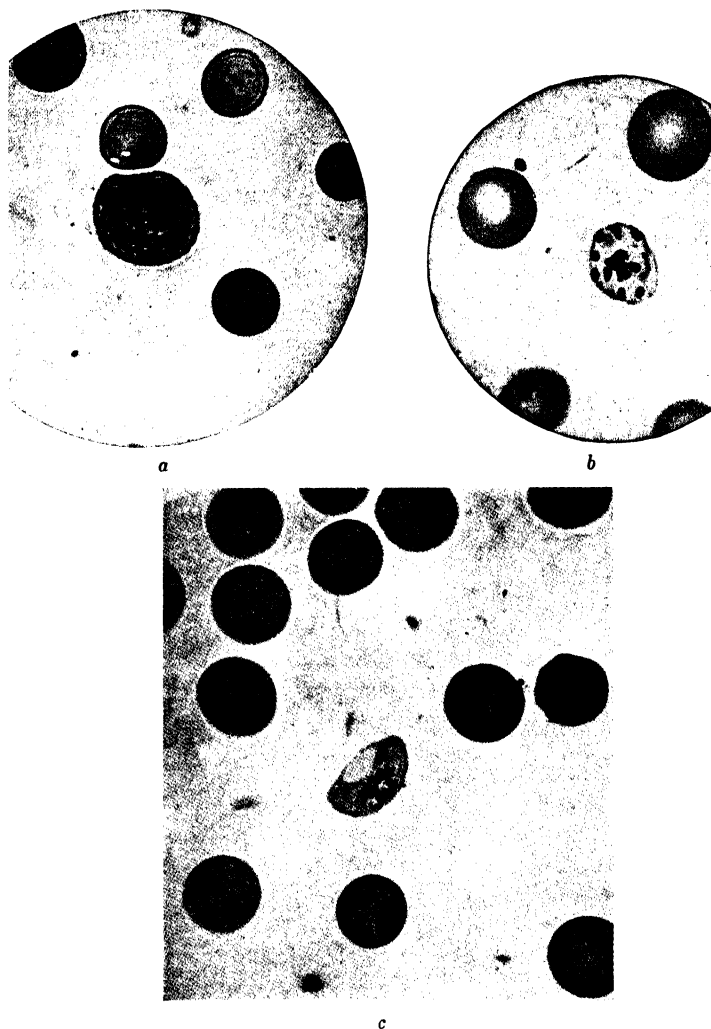


Fig. 384.—a, *Plasmodium vivax* (gamete); b, *P. vivax* (segmented); c, *P. falciparum*, showing crescentic form. (Army Medical School Collection, Washington, D. C.)

characteristics of the eggs, larvae and pupae as well as by the position and markings of the adult (Fig. 383). Students who are interested are referred to books on entomology.<sup>8</sup>

**Diagnosis of Malaria.**—The laboratory diagnosis of malaria is made by smearing a drop of the patient's blood evenly and thinly over a slide. It is stained with Wright's or Giemsa's stain or any of the group of polychrome stains known as Romanowsky stains which are valuable for such work. The protozoa can be seen in the blood cells and, depending on the stage of their development in the red cells may have any of the forms shown in Figures 381 and 384.

#### CLASS SARCODINA; THE ENDAMOEBAE

The endamebae or intestinal amebae constitute one genus of the large group of organisms (Sarcodina) which includes the ordinary, free-living amebae, such as *Amoeba proteus* or *Arcella vulgaris*, which every student of elementary biology has seen. Like ordinary amebae, the endamebae have no fixed form, but continually change their shape from round or oval to very irregular forms, with lobules and elongated portions protruding from various parts of the cell. They are about 30 microns in diameter. They move about by means of the protrusions, which are called pseudopodia (*pseudo* = like; *pod* = foot). In many respects amebae are like the leukocytes of the blood, especially in manner of engulfing food, moving, and reactivity to various stimuli such as foreign particles. Several species of amebae live in the human intestine. The most dangerous type is called *Endamoeba histolytica* (*histo* = tissue; *lytic* = dissolving). Others are *Endamoeba coli*, *Endolimax nana* and *Iodamoeba williamsi*. The last three are harmless but may be confused with *E. histolytica*.

**Amebic Dysentery.**—When human beings are infected with *Endamoeba histolytica*, the organisms establish themselves in the intestine. They attack the cells which line the colon and also ingest red blood corpuscles. They dissolve the tissues by means of a lytic enzyme. They take the corpuscles into their bodies in much the same manner as do the leukocytes. As a result of the irritating and destructive action of these parasites upon the intestine, a severe diarrhea develops and extensive ulcerations may form in the large bowel. Blood is found in the stools. If allowed to extend their activities, the parasites sometimes penetrate the lining of the bowel and cause perforations of the intestinal wall. The patient may then die of peritonitis caused by the bacteria in the feces. The amebae get into the blood vessels and are carried into the liver, lung, brain or other organs, excavating large cavities wherever they locate (Fig. 385).

*Transmission of Amebic Dysentery.*—During the acute diarrheic stage of the disease the parasites may sometimes be found in the feces, if these are examined while fresh and warm, in an actively

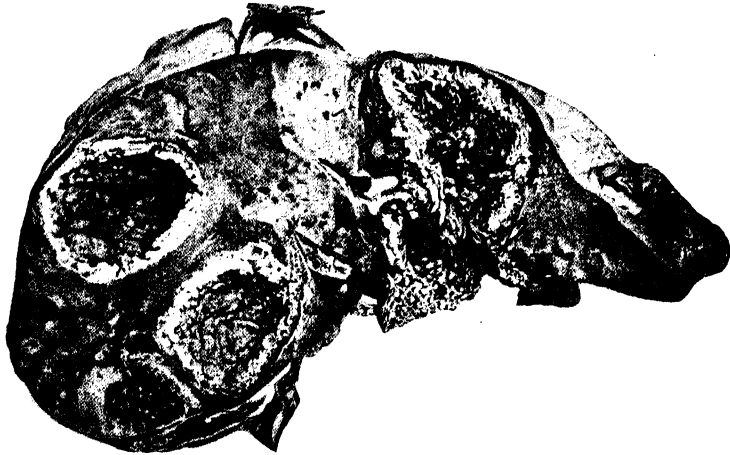


Fig. 385.—Multiple amebic abscesses of liver. (Mense's Handb. d. Tropenkrankheiten.)

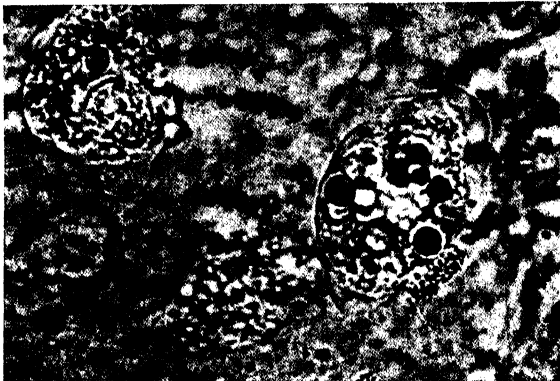


Fig. 386.—*Endamoeba histolytica* (trophozoite or active form). (Army Medical School Collection, Washington, D. C.)

motile, relatively fragile, vegetative or trophozoite stage (Fig. 386). As the disease becomes more chronic, and intestinal motion less rapid, they are found only in an inactive, resistant, cyst form



(Fig. 387). These cysts are often passed with the feces in large numbers. They can remain alive for days in moist feces or in damp, polluted soil or water. Anything contaminated with feces containing these cysts may therefore transmit the disease. Drought and sunlight are very unfavorable to the cysts. The trophozoites are not infectious as they are killed by gastric secretions. The cysts resist the action of the stomach and the cyst wall is digested by trypsin in the intestine, permitting the parasites to grow and invade the mucous membranes. Acute cases are, therefore, less apt to spread the disease than carriers. In the Orient and other places where fresh sewage and human feces are used as fertilizer for truck gardening, fresh vegetables such as lettuce, celery and the like are best left alone. The mere absence of clinical dysentery from the

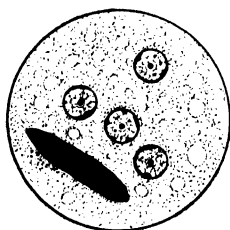


Fig. 387.—*Endamoeba histolytica* (cyst or dormant form). (From Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

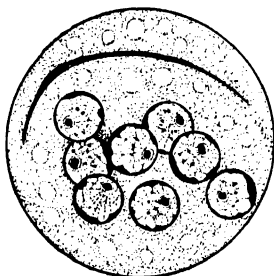


Fig. 388.—*Endamoeba coli* (cyst). (From Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

populations furnishing such fertilizers is no safeguard against the disease, since carriers of *Endamoeba histolytica* are common. It has recently been shown that they are not at all infrequent among people in various parts of the United States. A severe outbreak of the disease occurred in Chicago in 1933 due to accidental contamination of water by faulty sewer connections.

*Laboratory Diagnosis of Amebic Dysentery.*—As has been pointed out, during the acute stages the parasites appear in the feces in an active but very fragile form. If the stool is fresh and the specimen is kept at body temperature by means of an electrically warmed, microscope stage incubator, they may be observed directly. They are characterized by the presence within them of red corpuscles. In the later stages of the disease the cysts may be found in older specimens. These are about 15 microns in diameter. The

cysts of *Endamoeba histolytica* are characterized by the presence of four or less nuclei. They may thus be distinguished from the cysts

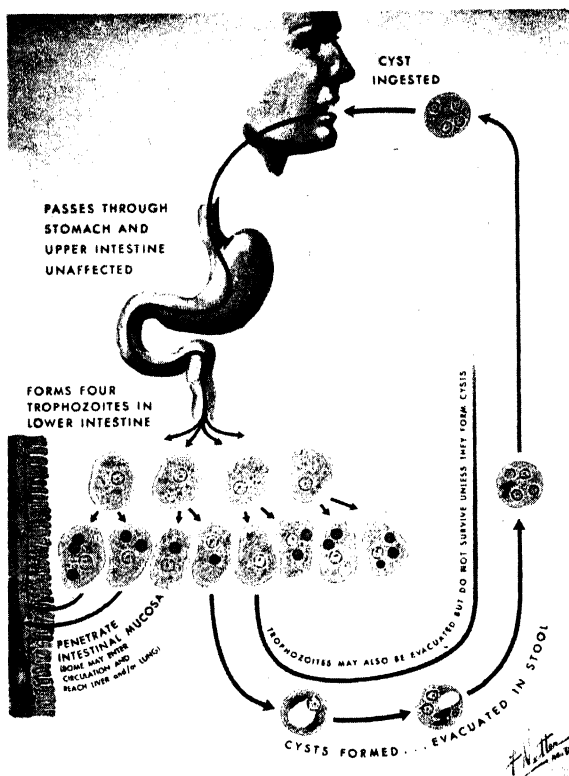


Fig. 389.—Life cycle of the *Endamoeba histolytica*. The cysts are swallowed and pass intact through the stomach and upper part of the intestine. In the lower part of the small intestine each cyst forms four trophozoites and these divide and multiply by fission. These trophozoites are motile and ingest red blood cells. Some of them penetrate the mucosa of the intestine by means of a lytic ferment which they secrete; ulcers are thus formed. Occasionally they may be carried by the blood stream to the liver where they form abscesses. Other trophozoites form cysts which pass out in the stool. These cysts, in their first phase, are uninuclear but later they form two and then four nuclei. Their large food vacuole is absorbed. If the bowels are very active, some of the trophozoites also are excreted. These may perish, or form cysts outside the body and survive. (Courtesy of Sharpe & Dohme, Inc., Phila., Pa.)

of a similar, but harmless, organism called *Endamoeba coli*. The mature cysts of the latter are usually larger than those of the former and

are further distinguished by the presence of more than four (usually eight) nuclei (Fig. 388). *Endamoeba coli* is often found in the stools of normal people and usually has no pathological or sanitary significance.

Diagnosis may also be made by cultivation of the parasites on Boeck's medium<sup>9\*</sup> or by means of complement fixation tests. The most effective drug for treatment is yatren. Carbasone and emetine are also used.

### CLASS MASTIGOPHORA

**Giardia Lamblia.**—These organisms have a very characteristic appearance and are found in all parts of the world (see Fig. 390). They were probably first observed by Leeuwenhoek in his own stools. In size they compare with the larger leukocytes. They live

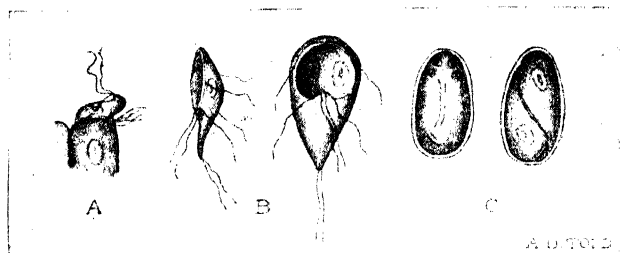


Fig. 390.—*Giardia lamblia*, the most prevalent intestinal flagellate: A, Flagellate form attached to the top of intestinal epithelial cell ( $\times 500$ ); B, flagellate form, side and ventral views ( $\times 1000$ ); C, cysts, frequently seen in the feces ( $\times 1000$ ). (After Chandler: A following Grassi and Schewiakoff, B and C following Wenyon.)

in the small intestine and fasten themselves to the cells lining it by means of a sucker-like depression, absorbing the body fluids. Like the amebae, they can form rather resistant cysts which are disseminated by any material recently polluted with feces. *Giardia* may cause an irritation of the intestine sometimes resulting in diarrhea. The organisms are, however, often found in normal people and animals. The giardias are not very dangerous organisms. Diagnosis may be made by examination of the stools with the microscope. The cysts have a distinctive appearance (see Fig. 390).

**Trichomonas hominis.**—These organisms are pear-shaped, flagellated protozoa which are, in general, a little smaller than the

\* Desiccated "Entamoeba Medium," ready for use, may be obtained from the Difco Laboratories, Detroit, Michigan.

giardias (Fig. 391). They probably do not cause any severe disturbances in the intestine. Cysts are not formed. The trophozoites are quite resistant, however, and serve as a means of transmission. They are found, not infrequently, in normal persons.

*Trichomonas vaginalis*, a similar species, is not uncommonly found in exudates from cases of vaginitis, but the etiological relationship is not entirely certain, as shown by Andrews in 1938.<sup>10</sup> The organism is commonly found in the normal vagina, and in the prostatic secretion of man.

*Trichomonas foetus*, closely related, infects the genitalia of cattle and produces vaginitis in cows. It is of economic importance due

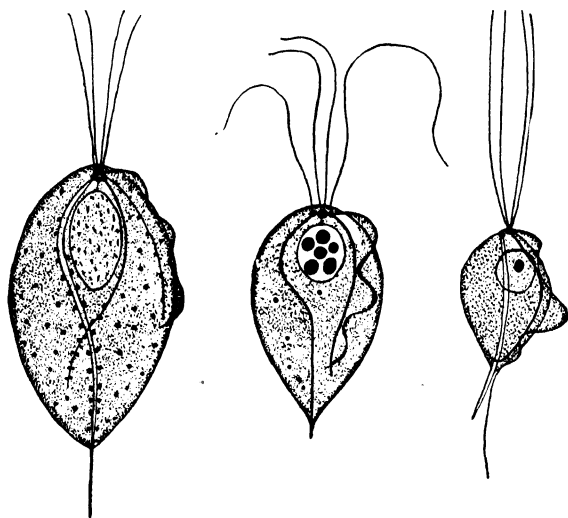


Fig. 391.—*Trichomonas vaginalis*, *Trichomonas buccalis*, *Trichomonas hominis* ( $\times 2000$ ). (Powell.)

to its relation to “delayed conception and other reproductive defects of susceptible cattle” (Andrews).

**The Trypanosomes.**—Adult trypanosomes are elongated, spindle-shaped organisms, extremely flexible and motile, and have the appearance shown in Figure 392; 4. They are from 15 microns to 30 microns in length and the diameter is about one-sixth of the length. In addition to a large and easily stained nucleus they have a flagellum which is attached, at the posterior end, to a complex granule called a blepharoplast and which extends to the anterior end of the body attached to the outer edge of an undu-

lating, keel-like membrane which traverses the length of the cell. Important species in human disease are those which cause African sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodesiense*). A serious disease of a somewhat similar nature in South America, called Chagas' disease, is also caused by trypanosomes called *T. cruzi*.

Many trypanosomes undergo four stages in development: the leishmania stage, the leptomonas stage, the crithidia stage, and the

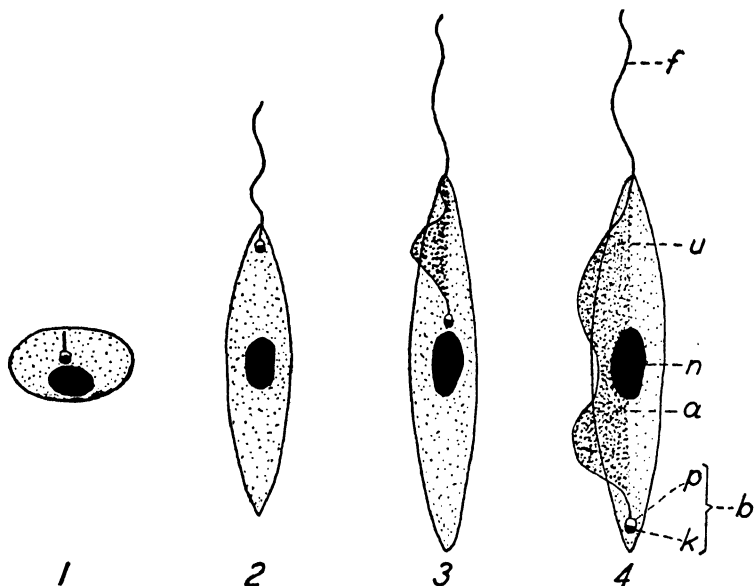


Fig. 392.—Developmental stages of typical trypanosome: 1, leishmania; 2, leptomonas; 3, crithidia; 4, trypanosome. a—axoneme; b—blepharoplast; f—flagellum; k—kinetoplast; n—nucleus; p—parabasal body; u—undulating membrane. (Culbertson, "Medical Parasitology," Columbia University Press, publishers.)

\* trypanosome stage. The first and last stages are usually spent in a vertebrate host; the other stages occur in invertebrate hosts. These four stages have not been observed to occur in all species of trypanosomes (Fig. 392). Characteristic differences of the four stages are shape, size, and development of the flagellum and membrane.

In sleeping sickness the adult organisms live in the blood and may be readily observed when stained by Wright's stain. They also occur in the lymph glands and spinal fluid. The trypanosomes of

African sleeping sickness are transmitted by the bite of a fly called the *tsetse fly* (*Glossina palpalis* and *Glossina morsitans*) (Fig. 393). The flies act as intermediate hosts of the parasite and the trypanosomes undergo developmental stages in the bodies of the insects. Tsetse flies occur only in Africa, and thrive especially in shaded areas along streams. Prevention of African sleeping sickness depends upon the elimination of the tsetse flies and certain wild animals which act as carriers of the trypanosomes. The untreated individual usually dies although he may live for months or

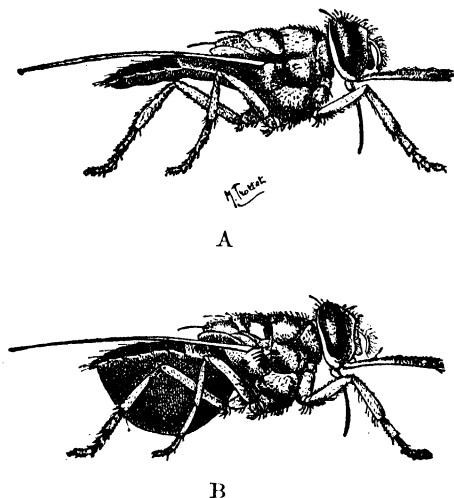


Fig. 393.—Tsetse flies. *A*, Before blood meal with abdomen undistended; *B*, after engorgement with blood. (Zinsser and Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

years in a dull or comatose state. Treatment with arsenicals (tryparsamide) or a complex drug called germanin is effective in some cases.

*Trypanosoma cruzi* is transmitted by the bite of a South American insect called *Triatoma megista* or, locally, a "barbeiro" (Fig. 394). There are many animal reservoirs, and other insect vectors.

There are several trypanosome diseases of animals which cause great economic loss. *Surra* of camels and horses is one of these. Another is *dourine* of horses. Due to the fact that the latter disease is transmitted by coitus, it is often spoken of as "equine syphilis." The organism causing it is called *Trypanosoma equiperdum*. The

United States Department of Agriculture has carried on a successful campaign to eliminate dourine among wild and domesticated horses, particularly in the western part of this country. An important means of diagnosis of the disease in the laboratory is by the use of the complement fixation test. Extracts of *T. equiperdum* are used as antigen. This procedure was developed largely by Dr. H. W. Schoening, who had charge of the work.<sup>11</sup> It has saved farmers and ranchers millions of dollars.

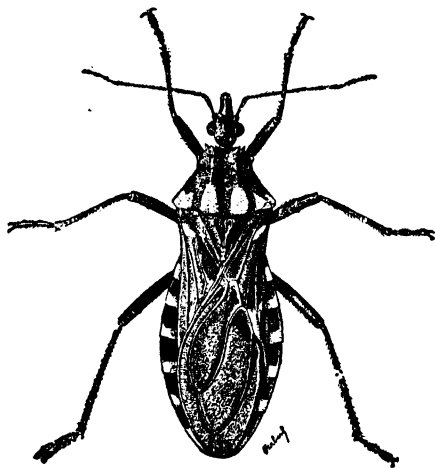


Fig. 394.—*Triatoma megista*, or "barbeiro," vector of Chagas' disease. (Zinsser and Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

#### THE LEISHMANIASIS

These organisms in some respects resemble the trypanosomes; that is, they undergo the first two of the four stages described for trypanosomes: the leishmania stage, seen in the tissues of the vertebrate host, and the leptomonas stage found chiefly in invertebrate hosts. Morphologically the three species causing human disease (*L. donovani*, *L. tropica* and *L. braziliensis*) are indistinguishable. The diseases caused are different, although the ulcerations caused by *L. tropica* and *L. braziliensis* are analogous in some respects.

*Leishmania donovani* causes kala-azar, a disease also known as visceral leishmaniasis. It occurs chiefly in the Orient, India, Mediterranean countries and other tropical countries of the eastern hemisphere. The leishmania stage occurs in the tissues of human

beings, commonly children, where it may be seen in preparations stained with Wright's stain. It is about 3 microns in diameter. The leptomonas stage will develop in cultures on N.N.N. medium\* or in sand flies (*Phlebotomus* flies, Fig. 395).

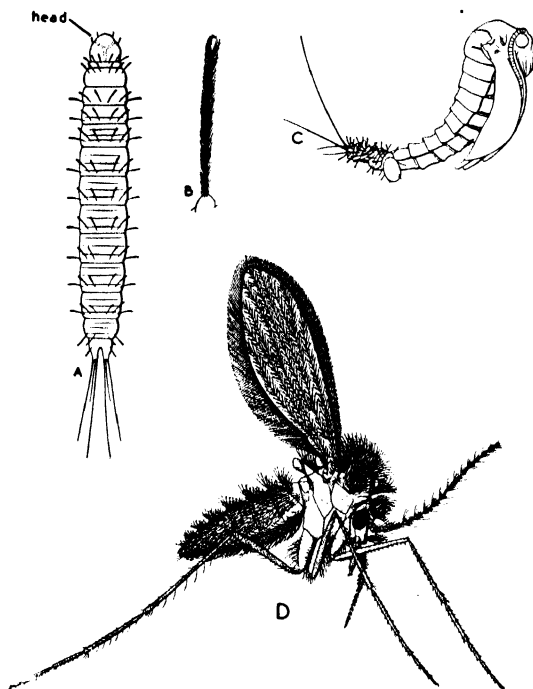


Fig. 395.—*Phlebotomus*. A and B, The last stage larva of *P. papatasi* and one of its characteristic hairs (after Grassi); C, Pupa of *Phlebotomus* species (original); D, Adult female of *P. papatasi* (original). A, C and D about  $\times 8$ ; B, much enlarged. (Smart, "A Handbook for the Identification of Insects of Medical Importance," British Museum.)

\* N.N.N. Medium (Nicolle, Novy and MacNeal).

Agar Base:

Agar .....	14 gm.
NaCl .....	6 gm.
Water .....	900 cc.

Melt agar in the boiling salt water and make up to 1000 cc. Distribute in 4 to 5 cc. amounts in tubes. Cool to  $45^{\circ}\text{C}$ . To each tube add about 1.5 cc. of defibrinated rabbit blood. Mix, and cool with ice water in a slanting position. Saturate the cotton plugs with sterile paraffine, or use cork stoppers.

Inoculum is placed in the fluid at the bottom of the slants. Incubate at  $22^{\circ}\text{C}$ . for 5 to 10 days.



The disease consists of a progressive and often chronic infection of the cells lining the blood vessels, especially in the spleen which becomes much enlarged. The liver, lymph glands and bone marrow also contain large numbers of the parasites. Diagnosis is often made by obtaining a bit of lymphatic tissue or bone marrow and examining stained smears of it. Cultivation is not difficult and will

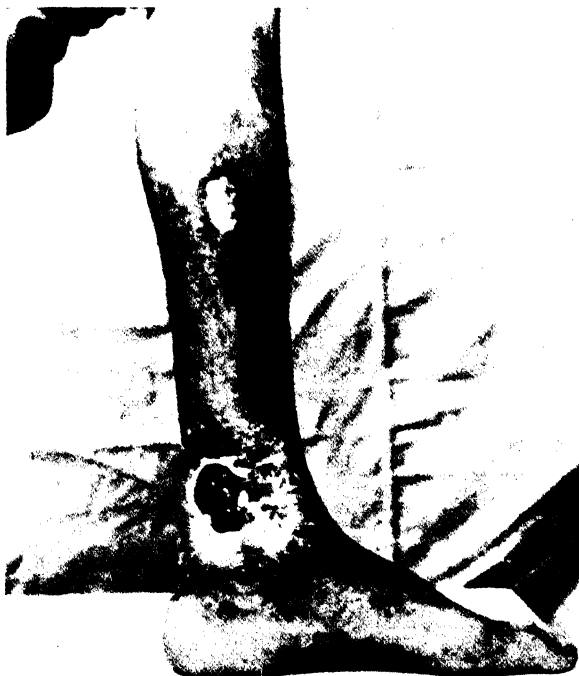


Fig. 396.—Cutaneous leishmaniasis; ulcer near a malleolus; above, scar from healed ulcer. (Culbertson, "Medical Parasitology," Columbia University Press, publishers.)

confirm a diagnosis based on smears. Acute infections often occur and are frequently fatal unless treated with antimony compounds, especially compounds of pentavalent antimony which are sometimes very effective. Recovery confers prolonged immunity. Vaccination is not effective.

The means of transmission of kala-azar is not entirely clear. Sand flies harbor the organisms after biting infected persons or feeding

on infected blood or tissue, and can be shown to be infectious if ground up and injected into susceptible animals or man. However, it is questioned by many whether the bite of infected sand flies actually transmits the disease under natural conditions. Some animals can be infected by feeding them the infected tissues. It is also possible that oral and nasal secretions of patients may transmit the disease since the organisms occur there.

*Leishmania tropica*, the cause of Oriental sore (Delhi boil, Aleppo boil, etc.) is very much like *L. donovani* in all respects. The



Fig. 397.—Facial espundia or American leishmaniasis, showing swelling of nose, lip and adjacent tissues. (Ruge, Mühlens, and Zur Verth, "Krankheiten und Hygiene der Warmen Länder.")

lesions caused by the organism are, however, confined entirely to the skin, especially of the face and extremities but not the palms or soles. The disease is very widespread in much the same general areas as kala-azar and the same uncertainty as to the means of dissemination exists. The same relations hold with respect to cultures and sand flies. Contact with infectious material such as actual sores, fomites, etc., may transfer the organisms from person to person. Dogs are susceptible and can carry the disease.

The sores may occur singly or in large numbers. They consist of ulcers with undermined edges, and several sores may coalesce into

ulcerative areas several inches across. Secondary infection by bacteria often occurs. Healing is slow but is the rule unless treatment with antimony compounds, locally or by injection, effects an earlier cure. Scars often result from the boils (Fig. 396).

Recovery results in immunity and, unlike kala-azar, vaccination by purposeful inoculation is an effective means of preventing extensive disease. It is used by natives of endemic areas for protecting children.

Diagnosis is generally not difficult since the parasites are readily seen in stained preparations made from the edges of the ulcers.

*Leishmania braziliensis* is so like *L. tropica* that some authorities consider it a variety of the latter. The disease caused by it (espundia, American leishmaniasis, uta, etc.) is in some respects like Oriental sore. The initial stages are similar but, whereas Oriental sore tends to heal spontaneously, espundia tends to progress. The organisms invade especially the tissues of the face, nose, mouth and tongue (Fig. 397). Enormous swelling, ulceration, and finally necrosis and destruction of tissues occurs, followed by secondary bacterial invasion and death. The organisms are seen in stained preparations from the ulcerated tissues.

The mode of transmission is unknown, but the same relation to sand flies and dogs holds as in the oriental leishmaniasis. Antimony compounds are effective as therapeutic agents.

#### CLASS CILIATA

*Balantidium coli*.—The class Ciliata is most frequently studied through the medium of the genus *Paramecium*. Paramecia are favorite classroom material because they are harmless, large, easily obtained, have well developed organelles, and illustrate many phenomena peculiar to protozoa. Every student of biology is familiar with the "slipper animalcule" and its lively and educational antics under the microscope. Less well known is a first cousin, the intestinal parasite *Balantidium coli*. This was discovered by Malmsten in 1857 in stools of dysentery patients and has since been shown to be a pathogenic organism.

The structure of *Balantidium coli* is consistent with its relationship to *Paramecium*, but it is ovoid in shape and about 60 microns long by about 50 microns wide (Fig. 398). The life cycle involves a trophozoite stage, active and pathogenic in the intestine of man and animals, and a cyst stage during which it is dormant and resistant to conditions in the outside world. It is primarily a parasite of pigs and is common in swine. Persons who are in occupational

contact with swine very frequently become infected although only a small proportion develop actual disease. Infection is transmitted in the cyst form, which develops in the intestine into the trophozoite. After varying periods cysts are found in the feces and any food, water, or other material contaminated with them may cause infection.

The disease is analogous in some respects to amebic dysentery, with ulcers formed in the intestinal mucosa. A proteolytic enzyme is probably produced by the parasite. The resulting dysentery is usually relatively mild although bloody stools are sometimes found.

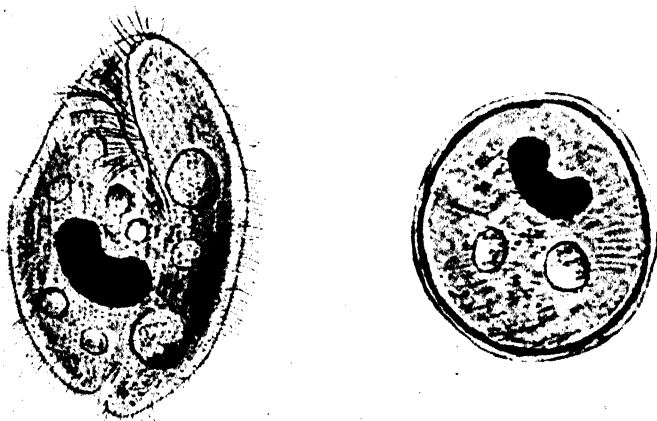


Fig. 398.—*Balantidium coli*. Drawing of trophozoite (left) and cyst (right) from specimens stained by iron-haematoxylin.  $\times 650$  app. (Lynch, "Protozoan Diseases of the Alimentary Tract," by permission of The Macmillan Co.)

Spontaneous cure is the rule. Diagnosis is made by observation of the characteristic cysts in the stools.

The parasite may be cultivated in Boeck's medium. Treatment is not usually needed but carbasone or Stovarsol may be used. Measures directed against infection with cysts of amebae will also help in preventing infection with *B. coli*, but must be extended to the avoidance of materials contaminated with swine feces.

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